STUDIES OF ENVIRONMENTAL FACTORS INFLUENCING IN VITRO GROWTH OF BASIDIOMYCETES AND THEIR ELABORATION OF BIOLOGICALLY ACTIVE SUBSTANCES

Thesis for the Degree of Ph. D.

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STUDIES OF ENVIRONMENTAL FACTORS INFLUENCING IN VITRO GROWTH OF BASIDIOMYCETES AND THEIR ELABORATION OF BIOLOGICALLY ACTIVE SUBSTANCES

By

Joseph Alfred Stevens

A THESIS

Submitted to the School for Advanced Graduate Studies of
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VITA

Joseph Alfred Stevens
candidate for the degree of
Doctor of Philosophy

Final Exam: November 11, 1957.

Dissertation: Studies of environmental factors influencing *in vitro* growth of Basidiomycetes and their elaboration of biologically active substances.

Outline of Studies:

Major subject: Microbiology
Minor subjects: Mycology, Parasitology

Biographical Items:

Born, January 3, 1927, Cleveland, Ohio

High School: Benedictine, Cleveland, Ohio, 1940-1944.

Undergraduate Studies: University of Dayton, Dayton, Ohio, 1944-1949, B. S.

Graduate Studies: Michigan State University, East Lansing, Michigan, 1949-1957. M. S. 1953.

Special Graduate Research Assistantship, 1952-1955.

Affiliations: Society of the Sigma Xi, Society of American Bacteriologists, Mycological Society of America, and the American Association for the Advancement of Science.

STUDIES OF ENVIRONMENTAL FACTORS INFLUENCING IN VITRO GROWTH OF BASIDIOMYCETES AND THEIR ELABORATION OF BIOLOGICALLY ACTIVE SUBSTANCES

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AN ABSTRACT

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ABSTRACT

In 1948, a program of screening Basidiomycetes for oncostatic principles was started as a cooperative project between Michigan State College and the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York City. The ultimate objective of the screening program was to obtain cultures of organisms showing oncostatic properties and to determine whether cultures of them would produce the active material under laboratory conditions.

Dried Boletus edulis var. pinicola sporophores were the first to demonstrate consistently a tumor-retarding activity. Attempts to secure a culture of this organism proved fruitful following numerous platings of Bavarian soil on nutrient agar plates. Preparations made from this organism, while grown under varying conditions, at times showed the presence of a tumor-retarding substance, but duplication of such results proved difficult, chiefly because of the slow growth-rate

Collybia radicata var. furfuracea was the next organism studied. Various nutrilites, and carbon and nitrogen sources were studied as they affected the growth of mycelium and the elaboration of the tumor-retarding substances. Once again, several preparations made from this organism and/or its filtrates demonstrated tumor-retarding principles, but these results could not be duplicated consistently. The requirements of this organism with respect to the production of the tumor-retarding principle could not be established with certainty despite the fact that the requirements for ample mycelial growth were satisfied.

Calvatia maxima was the final organism investigated. A study of various temperatures and growth periods disclosed that the tumor inhibitor which was found in the sporophore of this organism could be produced consistently and at will by means of laboratory cultures.

The results of the screening program are also reported.

TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

LIST OF TABLES

LIST OF TABLES - Continued

TABLE

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Knowledge of tumor-retarding properties of plants and their products is limited. It was not until recently that studies of plant derivatives in relation to tumor inhibition have been made.

Tne primary purpose of this investigation was to elucidate a portion of the nutritional requirements of *Collybia radicata var. furfuracea* and to study additional aspects of its physiology as they affected the elaboration of an oncostatic principle *in vitro*. The nutritional study included the characterization of essential nutrients for growth in a semi-synthetic medium. This involved various nitrogen sources, carbon sources, and nutrilites. Further physiological aspects pertained to the study of conditions required for growth, pH optima, and temperature optima, as they accompanied the production of the tumor-retarding principle.

An ancillary activity in the course of this investigation was the screening of extracts of Basidiomycete fruiting bodies for tumor-retarding action; the results will be reported here in part.

The ultimate objective of this work is to provide a basis for further investigation of the Basidiomycetes with the goal of establishing means of producing, *in vitro*, further agents active against tumors.

General Review of Literature and History

Long before the age of antibiotics, certain investigators reported the inhibition of living organisms by others. Brunel (1951) reviewed antibiosis from the time of Pasteur to Fleming.

Following the advent of penicillin many investigators turned to screening additional fungi for antibacterial substances. A few sought out members of the higher fungi for their investigations. Among these, Wilkins and Harris (1944) in England and later Wilkins (1945-1948) screened the sporophore extracts and culture broths of 900 species for activity against Escherichia coli and Micrococcus pyogenes var. aureus. They reported that 35 per cent of the Ascomycetes and 25 per cent of the Basidiomycetes of the total investigated had some degree of antibacterial activity. In addition, they noted that the composition of the substrate, whether natural or artificial, was the most important factor in producing an antibiotic. Mathieson (1946) reported similar findings employing Basidiomycetes gathered in Victoria. Bose (1945, 1947) discussed the presence of antibacterial substances in Polypores. Hollande (1945), reported in France that clitocybine, an antibiotic substance isolated from Clitocybe gigantea var. candida, was active against several bacteria, including Mycobacterium tuberculosis, Salmonella typhosa and Brucella abortus. Furthermore, he reported that positive therapeutic results were obtained employing guinea pigs infected with M. tuberculosis. Contrary to these findings, Robbins, Hervey and Kavanaugh (1946) observed activity by many Basidiomycetes

against M. pyogenes var. aureus in vitro but none in vivo. In a subsequent paper, Hervey (1947) mentioned that sometimes a negative correlation existed between growth of the Basidiomycete and production of the antibiotic principle.

Robbins (1953) reviewed the work accomplished by his co-workers and himself during the seven previous years on the presence of antibacterial substances in Basidiomycetes. This included papers by Robbins et al. (1947a, 1947b), Anchel et al. (1948, 1950, and 1952) and Kavanagh et al. (1949, 1950a, and 1950b). Melin et al. (1947) reported an antibiotic agent to be found in the substrate of cultures of the genus Marasmius, while Dorey et al. (1951) reported similar findings concerning Coprinus quadrifidus.

Following these screening tests, studies of individual species were made, and isolation of the various basidiomycetous antibiotics was attempted. Nancy Atkinson (1946a, and 1946b), following her screening of 200 species, worked on two different substances she obtained from Cortinarius rotundisporus and Psalliota xanthoderma. She found that sporophore extracts of these two organisms were active against a wide range of bacteria, that the activity of both extracts was unaffected by the presence of 10 per cent serum and the toxicity to animals was low. Later (1954) she reported the attempted purification of the active principles from aqueous sporophore extracts by paper chromatography. The erratic results obtained were proved to be due to exposure of the extracts to daylight. She also reported that ultraviolet light inactivated one of the anitbiotics, psalliotin.

Anchel, Polatnick and Kavanaugh (1950), while working with Poria tenuis, P. corticola and an unidentified Basidiomycete, isolated two closely related, highly unsaturated and highly unstable antibiotic substances from each of these organisms.

Anchel (1952) characterized the antibiotic drosophilin A (produced by Drosophila subatrata) as p-methoxy-tetrachlorophenol. In further studies, Anchel et al. (1955) compared its action with that of phygon, spergon, pentachlorophenol, hydroquinone, and p-methoxyphenol in tests against bacteria and fungi. It was more effective against fungi than bacteria, and the authors concluded that it was a fairly good anti-fungal agent.

As was inevitable, the scope of the investigators broadened to cover antifungal, antiviral, and anti-tumor agents. Utech and Johnson (1950) screened growth products, expressed juices, or water extracts of bacteria and fungi for their ability to inactivate tobacco mosaic virus. All the Basidiomycetes tested were active. These workers reported also that varying the cultural conditions caused a variation in the production of the inactivators. They concluded that, generally speaking, the temperature at which the organism grew best gave maximum production of the inactivators. Their attempts at chemical isolation were rather unsuccessful.

Reilly and Stock (1951) reported that extracts from the culture filtrates and mycelial pads of Aspergillus fumigatus inhibited the growth of Crocker mouse sarcoma 180 in mice. Additional work by Peterman, Hamilton and Reilly (1952) revealed the active portion to be

toxic and either a highly basic protein, or group of proteins, with isoelectric points near pH 10.0.

Stock et al. (1949) reported that a material from cultures of aspergilli had shown appreciable ability to inhibit Crocker mouse sarcoma 180.

Stock et al. (1954) reported that a crude filtrate from a culture of a Streptomyces sp. possessed potent anti-tumor activity. The active substance was characterized as O-diazoacetyl-L-serine. The crystalline antibiotic was found to be effective against sarcoma 180 in amounts as low as 1-2 mg./kg./day. Ehrlich et al. (1954) reported a correlation between the inhibition of sarcome 180 and the inhibition of a yeast, Kloeckera brevis, by azaserine.

Löfgren, Lüning, and Hedström (1954) reported the isolation and determination of the structure of nebularine from Clitocybe nebularis. This substance had a very selective bacterial spectrum, inhibiting only members of the genus Mycobacterium. However, the investigators added that in tissue culture tests, the substance retarded cell growth of sarcoma 180. No effect on sarcoma 180 cells in mice was reported. The compound was characterized as 9-(D-ribofuranosyl)purine.

In 1948, a program of screening members of the Basidiomycetes for oncostatic principles was started as a cooperative project between Michigan State College and the Division of Experimental Chemotherapy, Sloan Kettering Institute for Cancer Research, New York. The samples were tested for activity against Crocker mouse sarcoma 180 in the manner described by Stock and Rhoads (1949), Stock (1950), and Clarke (1955).

Among the fruiting body extracts screened (at that time), those of dried Boletus edulis var. pinicola consistently demonstrated a tumor-retarding activity. The ultimate objective of the screening program was to obtain cultures of organisms showing oncostatic properties and to determine whether cultures of them would produce the active principle under laboratory conditions.

Spores of B. edulis var. pinicola were obtained from habitats in Central Europe and attempts were made to germinate them on the medium and in the manner suggested by Khudiakov and Vozniakovskaia (1951). This was unsuccessful. Soil samples were then obtained from an area where this organism fructified profusely. Following numerous platings, an organism was isolated whose characteristics were not unlike those attributed to B. edulis by Melin (1921 and 1923). This culture was designated 288j.

CHAPTER II

METHOD OF BIOLOGICAL ASSAY

Experimental Details

The tumor-retarding activity of the prepared samples was determined at the Division of Experimental Chemotherapy of the Sloan-Kettering Institute for Cancer Research using the in vivo assay method reported by Stock and Rhoads (1949), Stock (1950), and Clarke (1955).

Preliminary to the inhibition test, the toxicity of the sample to mice was determined by administering a single dose of the sample at various levels. The maximum tolerated level guided in selecting the dosage for the inhibition test.

Five female Swiss albino mice weighing 18-22 grams were used for each bio-assay. Uniformly cut sections of Crocker mouse sarcoma 180 (ca. 5 mg. wet weight) were implanted subcutaneously by trocar in the right axillary region. Therapy was initiated 24 hours after the implantation and continued for seven successive days. The sample being tested was usually injected into the peritoneal cavity. The injections were administered twice daily. On the day following the last injection each mouse was weighed and the change in weight from the day of implantation was recorded. The weight change for each member of the group was averaged and the resulting figure was considered representative for that group. Tumor diameters were measured through the skin by means

of calipers. The largest diameter was selected for one measurement, and an axis perpendicular to it was taken for a second measurement. The two diameters were averaged for each mouse to obtain an average tumor diameter. This parameter was averaged for the group and will be referred to as the tumor diameter of the treated mice in future discussions.

A second group of five mice was employed as controls. To these was administered an equivalent amount of physiological saline twice daily for the seven day period.

The criterion for the determination of inhibition was based upon a comparison between the development of the tumors in the treated animals and those in the untreated (saline injected) mice. The weight change of the treated animals was also compared with the weight change in the control group.

Interpretations of Assays

The results of the single-dose toxicity tests were reported as a number within the range of 0.5 and 5.0 (at 0.5 increments). The smaller number represented extreme toxicity accompanying a single dose of 0.5 ml. and 5.0 represented non-toxicity accompanying a single dose of 5.0 ml. Numbers between these two arbitrary limits represented gradations of toxicity.

The weight change was reported as the ratio of the average weight change in the treated animals to the average weight change in the control group.

The following arbitrary scale was employed in the inhibition test for grading the effects of the samples for tumor-retarding activity:

Marked inhibition (+)	Tumor diameter of treated group less than 25 per cent of the value for the control group.
Good inhibition ($\pm^{+}$)	Tumor diameter of treated group between 25 and 50 per cent of the value for the control group.
Slight inhibition ($\pm^{-}$) ...	Tumor diameter of treated group between 50 and 75 per cent of the value for the control group.
No effect (-)	Tumor diameter of treated group greater than 75 per cent of the value for the control group.
Questionable effect (?) ..	This designation was used when three or more mice died during the course of the inhibition test. Most of the samples giving a questionable effect were re-tested at a greater dilution.

Discussion of Assay

According to Stock (1954), sarcoma 180 was chosen as the assay tumor because of its nearly 100 per cent transplantability, low regression rate, rapid growth, lack of specific host requirement and its apparent intermediate sensitivity to several adverse agents. Up to the time of his report, approximately 9500 compounds and many natural products had been screened against this tumor, with 12 of these giving a (+) result. He further reported that confidence in the assay arises from the fact that all 12 of these compounds have shown benefits in the case of certain forms of human cancer.

On the other hand this assay had a few objectionable operational features when it was employed as a standard laboratory tool for this project. Although the actual assay only extends for a period of seven days, additional time was required for toxicity studies, for shipping the samples to New York City and for mailing the results back to this laboratory. Under the most favorable circumstances, this amounts to a minimum time interval of three weeks. Such a time interval between preparation, testing and receiving the results eliminates the possibility of more samples being prepared in a given period. It also introduces the possibility of the active principle being destroyed or diminished owing to such factors as time and temperature variation between the preparation and the testing of a sample.

Toxicity manifestations create another objection. Certain preparations demonstrated a single-dose toxicity not normally associated with the active principle. This necessitated the giving of a small dose with the possibility of an ensuing negative result because of dilution. The test, therefore, becomes merely a qualitative assay showing only the presence or absence of an active principle. This situation is compensated by the knowledge of the tumor diameters.

Another objection arose when it became difficult to distinguish between significant inhibition and normal biological variation. As a result of this condition a "significant trend" will be reported in a few cases, although sufficient activity did not exist for the sample to be graded "positive." It would be very desirable to have an assay

which did not include the objections mentioned above. A chemical, bacteriological or seed assay may possibly contain the answer. However, at present, no such generally approved method has been devised.

CHAPTER III

INTRODUCTORY EXPERIMENTS: EXPERIMENTAL WORK WITH BASIDIOMYCETE 288j

The organism designated by the code number 288j was isolated late in 1952 by sprinkling one of many soil samples received from Bavaria on the surface of a nutrient agar plate. These soil samples were obtained from habitats that normally gave rise exclusively to many Boletus edulis var. pinicola fruiting bodies annually. The soil samples received were collected at an elevation of approximately 2500 feet, latitude 49°, longitude 13° east of Greenwich. The habitats are spruce-fir forests with occasional pines and deciduous hardwoods.

The organism was isolated and grew at 27°C. on a modification of Czapek's (1902) medium, which henceforth will be called "medium A". The composition of medium A is as follows:

$MgSO_4$	0.5 gm.
KH_2PO_4	1.0 gm.
KCl	0.5 gm.
$FeSO_4$	0.01 gm.
Difco Bacto-Peptone	5.0 gms.
Dextrose	15.0 gms.
Sucrose	15.0 gms.
Difco Yeast Extract	5.0 gms.
Difco Agar	15.0 gms.
Distilled water to	1000 ml.
pH	5.6

A "medium B" was also used which has the same composition as medium A except that it lacks yeast extract. In addition, broth forms of media A and B were made by excluding agar from the formula.

Microscopically, the hyphae of 288j showed clamp connections, one of the identifying characteristics of Basidiomycetes. The culture isolated grew slowly: a small (3.0 mm.) pea-sized inoculum grew to an approximate size of 1.25 cm. in four weeks. Its appearance at first was undistinguished. However, after two months, the organism's appearance was that of a small mound, covered with short tapering tufts (see Figure 1). These corresponded to the characteristic tufts seen in the photographs of cultures of B. edulis grown by Melin (1921). Because of the similarities of growth-rate and appearance, combined with the knowledge concerning the source of the soil samples, the isolate was considered to be a culture of B. edulis var. pinicola.

The late Dr. E. A. Bessey, for many years head of the botany department of this institution, was consulted several times and agreed with this conclusion.

The next step was to attempt to grow this organism in greater quantity and in less time. The solution became evident when 3 mm. pieces of 288j were grown on the surface of nutrient agar in Petri dishes; in moving the pieces of 288j into the desired position on the plate, microscopically small fragments gave rise to entire new colonies. It followed logically that the organism should be broken up in a sterile Waring Blendor, using sterile distilled water, in much the same manner as described by Savage and Vander Brook (1946) and also advocated by Dorrell and Page (1947) in order to obtain a shorter lag period and a closer check of replicates. Burkholder and Sinnott (1945) stated that this type of submerged shake culture produced mycelial pellets which

Figure I. Boletus edulis, strain 288j, grown for 7 days on medium A agar (enlarged 7x).

were either globous or irregular, hirsute or smooth, and either hollow or solid depending on the size and type of inoculum.

The procedure for preparing this 'inoculum' was standardized in the following manner: Pea-sized pieces of 288j were grown on 12 slants of medium A for three weeks. The slant demonstrating a colony size closest to 15 mm. in diameter was chosen as most desirable for preparing the inoculum. The colony was removed aseptically from the agar surface and blended under sterile conditions in a Waring Blendor (Monel metal) with 50 ml. distilled water for two minutes. Ten ml. of this inoculum was the amount generally employed to inoculate 125 ml. of medium A broth in a 500-ml. Florence flask, or the surface of a Petri dish of solid medium A. At the end of eight days, the broth cultures (which were aerated in the manner suggested by Kluyver and Perquin (1933) by continuous agitation on a reciprocating shaking machine having a stroke of 0.5 inches and giving 100 one-inch excursions per minute) would appear to be 50 per cent filled with small spheres of the organism. In the case of Petri dishes inoculated with one ml. of the blended suspension, the surface of the agar was generally covered entirely with 288j mycelium within 14 days.

After the organism 288j had grown in 125 ml. of medium A for six days, the mycelia from two 500-ml. Florence flasks were separated from the broth portions. The broth portions were mixed, and two 85 ml. aliquants were removed. One of the aliquants was sterilized employing a ST (Hercules Filter Corporation) Seitz filter, and the other preserved by adding 0.85 ml. of a one-per cent merthiolate solution. The treated

solutions were aseptically poured into sterile serum bottles, labelled, and frozen before shipping. The mycelia, regardless of the amount, were blended with 85 ml. of distilled water, Seitz filtered, and bottled.

Forty-seven samples were prepared in this manner. However, a few variations were attempted. The growth intervals extended from seven to 24 days, and one sample was blended with an ascorbic acid solution (one per cent) rather than distilled water.

None of the samples produced the desired activity. From the first group of samples, it was ascertained that there was nothing to be gained by sterilizing the samples by Seitz filtration rather than by adding merthiolate. Consequently, most of the samples were merthiolated.

Thirty-six of these 47 samples comprised an experiment to determine the effect of incubation time upon the elaboration of the active principle. A flask was removed from the shaking apparatus daily for 18 days, commencing with a seven-day growth period and terminating with a 24-day-growth-period culture. Each day a broth sample was prepared, and also a sample was made by blending the mycelium with 85 ml. distilled water. As mentioned previously, when tested all the results were negative. However, while the samples prepared from the seven-, eight-, and twelve to twenty-four-day old cultures produced no effect on the tumor, (i.e. the test and control tumors were approximately the same size), the samples prepared from the nine-, ten-, and eleven-day-old cultures had a tendency to depress the growth of the test tumor (though not sufficiently to give a reading of $\pm^{-}$). This tendency

in both the broth and mycelium-water samples later prompted the blending of the mycelium with the culture broth in an effort possibly to concentrate the active principle rather than dilute it by the addition of water.

Attempts were made to grow 288j in the liquid form of the synthetic (chemically-defined) medium (code letter J and described in Appendix) proposed by Khudiakov and Vozniakovskaia (1951) for growing four Boletus species. The growth in this medium was exceptionally slow. A growth period of 53 days was required to produce the same amount of 288j mycelium that was observed using medium A within eight days. This sample, mycelium blended with broth, also produced a negative result.

At this time, 288j was also grown in an altered version of medium A. The nitrogen and carbon ratios (normal being 1:10) were adjusted to be 1:5, 1:20, and 1:40. Two sets of these media were inoculated. One set was grown for 19 days and the second set for 83 days. The extracts were prepared by blending the mycelium with the culture broth. However, both sets were negative. The mycorrhizal relationships of many boleti have been recognized by, among others, Pennington (1908), Melin (1921, 1923), Melin and Nilsson (1952); in addition Rennerfelt (1950) reported about the growth of various fungi on sawdust. It was, therefore, decided to investigate the effect of a one-per cent birch sawdust extract, and a one-per cent fir and spruce sawdust extract. It was theorized that possibly these wood extracts might contain a substance which would support the elaboration of the oncostatic principle by the

organism, since species of this genus are known to grow in association with these trees.

On the first attempt, using 3.5 ml. of a one-per cent fir and spruce sawdust extract added to 125 ml. of liquid medium A, a ± result was obtained. Culture 288j was grown for 21 days in this medium, and the sample was prepared by blending the mycelium with the culture medium. Duplications and variations (21 in all), however, failed to reproduce the initial result. The symbiotic effect of organisms had been known for some time. It was decided to grow 288j in combination with other organisms isolated from the same soil because of the work reported by Zeller and Schmitz (1919) and Porter (1932) on the stimulatory and antagonistic effects which different microorganisms have upon fungi. For this purpose, three Streptomyces species were chosen: #252(0)$_2$, #288k, and #311. These were inoculated singly, along with 288j, into liquid medium A and grown for 21 days in the usual manner. The results were all negative. It was then decided to investigate the growth of 288j on solid media. Six Pyrex preparation dishes, containing 100 ml. of medium A agar were inoculated in the manner previously described. These were grown for 24, 44, 47, 51, 54, and 62 days at 27°C. The samples of the cultures of the first five growth periods were prepared in the following manner: 1) the mycelial mat was removed and blended with 85 ml. distilled water, filtered, sterilized and bottled, 2) the agar on which the organism had grown was extracted using 170 ml. distilled water, sterilized and bottled. The culture representing the longest growth period was made into one sample by blending the mycelial mat together

with the agar with 170 ml. distilled water; the preparation was sterilized and bottled. All of these 11 samples produced negative results.

In an experiment using positively ionized air (produced by a "Wesix" ion generator suspended in a flask) above 288j growing on the surface of agar one positive result was obtained. Two samples were prepared as described above, one of the mycelium, and the second an extract of the solid medium. The latter sample produced a $\pm^-$ effect. These experiments were repeated later, but with negative results.

Sterile quartz sand in a Petri dish thoroughly moistened with medium A broth was inoculated with a pea-sized piece of 288j mycelium, and incubated at 27°C for 60 days. At the end of this time a sample was prepared by extracting the mycelium with water. Although the Sloan-Kettering method of evaluating tumor inhibitors showed a negative result, a significant retardation of the tumor was noted.

At this time, 288j was also grown on one-inch cubes of du Pont cellulose sponge bathed in medium A broth in Pyrex "preparation dishes". These dishes were taped with one-inch masking tape to prevent contamination and the escape of moisture, and incubated at 27°C. The samples were prepared by blending the mycelium with the culture broth. Three samples, 8919, 8920, and 8921 (see Table 1) were prepared from cultures grown in this manner for 60 days. The appearance of the broth of sample 8919 was normal, i.e. golden-yellow. Sample 8920 was slightly darkened, and sample 8921 was dark brown in color. All three of these samples produced a $\pm^-$ effect. Two samples (8956 and 8957) which were inoculated at the same time as 8919 were prepared 30 days later, but

produced negative results. One of these (8957) also demonstrated slight pigment formation. Eight additional samples were prepared in an attempt to reproduce the initial positive results with no avail. These covered a growth period which ranged from 26 to 75 days.

Three test tubes, about two months after 288j was first isolated, showed rather unusual growth. The colonies in these tubes developed long tapering tufts, quite unlike those ordinarily seen. Their color was light peach, and their length approximately one centimeter. The colonies grew rapidly upon isolation, and gave off an odor not unlike that associated with Saccharomyces cerevisiae and other common yeasts. All attempts to isolate or observe a contaminant from these cultures ended negatively, and to this day the cultures designated 288jb, 288jc, and 288jd (see Figure II) grow as pure cultures. These cultures were shown to the late Dr. E. A. Bessey, and after extensive observation he concluded that, even though he observed structures similar to yeast cells, the cultures were to be regarded as spontaneous mutants of 288j. Accordingly, seven samples were prepared employing 288jb grown in shake culture and also on cellulose sponge cubes, but the desired effect was lacking. Five samples incorporating growths of 288jc and 288jd gave similar negative results.

The association of the yeast-like odor with the cultures of 288jb, 288jc, and 288jd recalled that Fries (1941, and 1943) has shown that some yeasts exerted a stimulative action on spore germination of Basidiomycetes. Eleven yeasts which were isolated from the Bavarian soil that originally yielded culture 288j were investigated for any

Figure II. Boletus edulis, mutant strain 288jb, grown for 1 day on medium A agar (enlarged 3x).

stimulatory effect they might have upon this organism. The yeasts were inoculated in the immediate vicinity of seven-day-old cultures of 288j, but no stimulatory effect was observed. In addition, the eleven yeasts were grown separately in medium A broth for four days at room temperature on the shaker. None of the samples produced from these cultures gave rise to a tumor-retarding principle.

Table 1

Boletus edulis, strain 288j, grown on cellulose sponge in a pool of medium A.

Sample Number	Age of Culture	Color of Broth	Mouse Toxicity*	Dose: cc 2x Daily	Effect	Tumor diam. in cm./diam of Control Tumor	Wt. change in gm./wt. Change of Control
8919	60 days	Yellow	> 5.0	0.8-0.4	±	0.69/1.11	-1.0/-1.5
8920	60 days	Light brown	5.0	0.5-0.3	±	0.78/1.11	-0.5/-1.5
8921	60 days	Dark brown	> 5.0	0.8-0.4	±	0.75/1.11	-3.0/-1.5
8956	90 days	Yellow	5.0	0.6	-	1.13/1.00	-1.5/-1.0
8957	90 days	Light brown	5.0	0.8	-	1.18/1.00	-1.5/-1.0

*The information in this as well as in the following columns was obtained, in tests of the mycelium extracted with culture media, at the Division of Experimental Chemotherapy of the Sloan-Kettering Institute for Cancer Research.

CHAPTER IV

EXPERIMENTS WITH *COLLYBIA RADICATA VAR. FURFURACEA*

Preliminary Experiments

Owing to the slow rate of growth of the organism 288j and its apparent inconsistency in producing the tumor-retarding principle, it was decided to investigate *in vitro* cultures of Basidiomycetes which grew more rapidly.

After we had obtained tumor retardation with water extracts of fresh *Collybia radicata var. furfuracea* sporophores, a flask containing culture broth A was inoculated with a culture of this organism (code designation #406f) and grown on a shaker at room temperature until the entire flask was filled with mycelium (30 days). At the end of this period, a sample was prepared (#9130) by blending the mycelium with the culture broth. The result (Table 2) indicated that the tumor-retarding principle was produced *in vitro*. A second sample was prepared in the same manner, this time employing a 21-day-old culture. This (#9158) also produced a $\pm^{-}$ result. A third sample was prepared, again in the same manner, but this time employing an 8-day-old culture. This sample (#9174) did not show the anti-tumor activity. A fourth sample was prepared, again in the same manner, this time using a 16-day-old culture. This sample (#9179) produced the tumor-retarding principle. Assuming that the nutritional requirements were adequately fulfilled, it was

Table 2

Elaboration of a tumor-retarding principle by Collybia radicata var. furfuracea #406f grown in shake culture at room temperature.

Sample Number	Strain	Age of Culture	Mouse Toxicity*	Dose: cc. 2x Daily	Effect	Tumor diam. in cm./ diam. of Control Tumor	Wt. Change in gm./ wt. Change of Control
9130	406f	30 days	3.0	0.5	±	0.88/1.38	-2.0/-1.5
9158	406f	21 days	> 5.0	0.8	±	1.05/1.42	-1.5/-0.5
9174	406f	8 days	5.0	0.5	-	0.90/0.93	-2.0/-1.5
9179	406f	16 days	5.0	0.8	±	0.80/1.14	0/+1.0
9232	406b	20 days	> 5.0	0.8	±	0.72/1.03	-3.5/0

* The information in this as well as in the following columns was obtained, in tests of the mycelium extracted with culture media, at the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research.

decided to find out whether there was a time factor involved in the production of the active principle.

Seven flasks of culture broth A were inoculated with 406f and grown on the shaker at room temperature. A flask was taken off the shaker, and a sample prepared, at the end of 3, 6, 9, 16, 20, 23, and 27 days, respectively. None of these samples demonstrated the presence of the active material.

A carrot broth was also investigated for its ability to 1) support the growth of 406f and 2) produce the active principle. A sample produced from the mycelium and broth of said culture failed to indicate the presence of any active material. What was especially interesting about this sample was the fact that it was started from the same inoculum which produced the active principle in sample #9158 (Table 2). It can be seen that, even though the carrot medium supported the growth of the organism 406f, it failed in some way to stimulate the production of a tumor-retarding principle.

At this point it might be appropriate to explain the identification of organism 406f. The sporophore was found in Sanford Woodland, part of the Michigan State University campus. It was 25 cm. high, the cap light brown, velvety to semi-viscid, and 10 cm. across. The spores were white and measured 9-10 microns x 13.5-16.1 microns. It was tentatively identified as Collybia longipes, even though Dr. E. A. Bessey pointed out that C. longipes specimens are extremely rare in Michigan. This first identification was based upon three observations: 1) the cap was brown and velvety, rather than the beige-light tan and

viscid cap generally ascribed to C. radicata, 2) its height, and 3) the size of the spores was intermediate between the two species, and definitely closer to those of C. longipes. However, Smith (1949) makes note of the variety furfuracea in listing the characteristics of C. radicata and these fit the specimen perfectly. A discussion with Drs. Bessey and Smith concerning identical specimens convinced the author that the proper designation should be C. radicata var. furfuracea. Apparently, however, some European mycologists have circumvented this difficulty by placing the species radicata in the genus Mucidula, while retaining the species longipes in the genus Collybia.

Collybia radicata var. furfuracea #406f grew at temperatures ranging from 12° to 28°C., with the optimum temperature range between 20° and 26°C. This was determined by placing 7.0-mm. plugs on triplicate medium A agar plates at temperatures of 12, 16, 20, 24, 26, 26.5, 27, and 28 degrees C. and recording the diameter of each colony. The averaged resultant values are shown diagrammatically (Figure III).

A second specimen of C. radicata var. furfuracea obtained from the same area which yielded 406f and designated #467. Three samples were prepared using this new isolation but none were active.

Because of the stimulatory effects noted by Hawker (1936), Fries (1943), Melin (1946), and Oddoux (1953) when natural extracts are incorporated into basal media, a medium was formulated which contained 53 such water extracts. To the dry ingredients of medium A, 10 ml. of each extract was added, and the final volume brought up to one liter

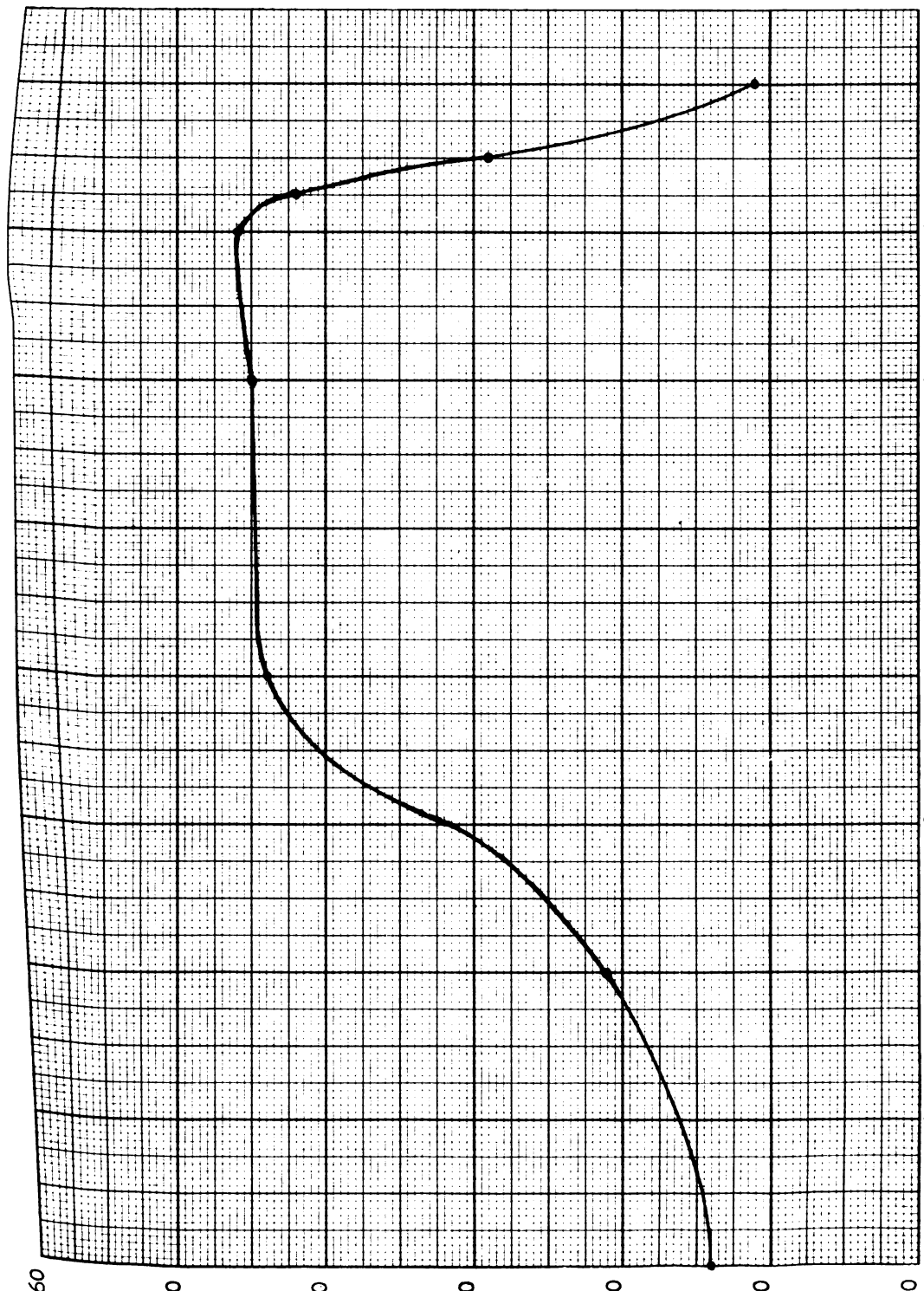

Figure III. Diameter of seven-day-old mycelial colonies of Collybia radicata var. furfuracea #406f grown on medium A agar plates.

by the addition of distilled water. This medium was designated Super A. The 53 extracts were prepared and chosen on the basis of previous reports of their stimulatory action as well as some which were chosen because of their natural associations or encounters with basidiomycetous mycelia in the forest soils. They included 10 per cent solutions of the following: wheat, barley, rye, lentil, white navy bean, and corn seeds; grass, hay, nettle, fresh alfalfa and alfalfa hay; maple, elm, and oak leaves, Douglas fir, spruce, and pine needles; plum, apple, cherry, strawberry, red raspberry, peach, blueberry, and coconut fruits; cocomut milk, spinach, asparagus, lettuce, corn, carrot, peas, and tomato vegetables; beef duodenum, liver, stomach lining, pancreas, and hog kidney*; Biopar A, B, C, D, E, F**; dried skim milk, dried egg yolk, corn steep water, dried yeast--strain G, Anheuser-Busch-, culture media of bacterium #457, Alternaria sp. #175A, Penicillium sp. #233K, Aspergillus sp. #458, and Fusarium sp. #314E.

Both 406f and 467 were grown in Super A medium, but the samples prepared from these cultures gave no indication of any tumor-retarding activity.

Since ionized air was found to have some apparent effect on the production of a tumor-retarding principle in a B. edulis culture (cf. p. 19), a similar attempt was made with culture 406f grown on the shaker in medium A, using positively as well as negatively ionized air. No active principle was found in either instance.

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*Viobin Corporation, Monticello, Illinois.

**Liver fractions, Armour and Company, Chicago, Illinois.

In trying to explain the inconsistent elaboration of the tumor-retarding principle by 406f, it was theorized that perhaps an accumulation of toxic metabolic products occurred in the medium in which the culture grew. The assumption was that these toxic products in the sample might interfere with the activity. In an attempt to test this possibility, three 500-ml. Florence flasks were modified by attaching an arm at a 45° angle at the base of the neck of each flask. At the point where the base of the armature met the Florence flask, a circular stainless-steel screen was sealed into permanent position. The purpose of this arrangement was to allow decanting the broth and replacing it with fresh sterile culture medium.

For the purposes of the experiment, the culture medium was changed every three days. The decanted broths from the three flasks were combined, and an 85 ml. aliquant was tested. Thirteen successive culture media were prepared. At the end of the thirteenth growth period the mycelium from the three flasks was removed and blended with 85 ml. of the last culture broth. With the exception of the sample prepared at the end of the twelfth growth period, all the broth samples were negative. The broth sample of the twelfth growth period as well as the mycelial extract made at the end of the thirteenth growth period produced a ± result.

Owing to the inconsistency of the elaboration of the tumor-retarding meterial, it was decided to investigate some of the other isolates obtained from the same pileus. Originally seven separate isolates had been obtained, and these were designated 406a, 406b, 406c, 406d, 406e,

406f, and 406g. Work was started with 406f because it was the fastest growing culture. Its relatively speedier growth-rate is unexplained since all seven isolations were from the same pileus. In checking the six remaining isolates, 12 samples were prepared from these cultures grown in four different media.* Only one of these, 406b (#9232 in Table 2) produced a positive result. When this isolate was again grown in the same manner, there was no indication of the presence of any tumor-retarding material.

When the production of the tumor-retarding principle by 406f and the other 406 isolates proved inconsistent, it was decided to investigate other sources of the same and similar species. The Centraalbureau voor Schimmelcultures Baarn (Holland) furnished cultures of Collybia longipes and Mucidula radicata (designated by our code numbers 451 and 450 respectively). The nomenclature of organism #450 (M. radicata) is European, and corresponds to the American Collybia radicata. Both of these cultures were grown on the shaker in medium A for 27 to 40 days, but no tumor-retarding principle was detected in any of the four samples prepared from these cultures. In addition, organisms 450 and 451 were grown in a malt extract broth for 68 days, and the latter in medium J for the same period of time, but no tumor-retarding principle was detected in any of the samples prepared from these cultures.

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*The four media employed (formulae in Appendix) were:
Medium A (Basal medium plus 0.5 per cent yeast extract)
Medium J (Khudiakov's synthetic medium)
Medium G (Sodium-caseinate medium)
Medium B (Basal medium)

CHAPTER V

THE EFFECTS OF NUTRILITES UPON *COLLYBIA RADICATA VAR. FURFURACEA*

Review of Literature

The term "nutrilite" was coined by Williams (1928). In a later paper, (1941), he discusses the development and defines the meaning of the term. It refers to a number of substances present in yeast extracts which were formerly known under various names. By definition it is now meant to "include those organic substances which in minute amounts function in a similar manner for *other types of organisms* as the vitamins function in *animal* nutrition."

Within the limits of this definition, we are able to include, besides some of the vitamins, such compounds as the nucleic acids and their derivatives.

It was in the same sense that Derrick (1949) employed this term. Williams (1941) went on to say, "The term 'nutrilite' like the term 'vitamin' is a convenient one for designating organic nutrients which are effective in minute amounts, but the distinction between an ordinary nutrient and a nutrilite is not, so far as we can see, a very fundamental one."

Some vitamins are known to play an important part in the growth of bacteria, yeasts, fungi, higher plants, and animals. B-vitamins may be either essential for growth, stimulatory, or inhibitory.

Countless papers have appeared on this subject, but only the literature dealing with members of the Basidiomycetes will be reviewed.

Fries (1938) reported that most members of the Polyporaceae require thiamine for growth, and that biotin and inositol had no effect upon this group. He also found that various bacterial culture extracts apparently contained a growth-stimulating agent which exerted, when used in very small quantities, a marked effect upon the growth of some polypores. The substance was identified as being, at least partially, aneurin (thiamine).

Rennerfelt (1944) while working with only a single member of this group, Fomes annosus, reported that it required thiamine for growth. He stated that the addition of 0.1 gamma of thiamine per 50 ml. of medium produced optimal growth. Yeast extract, he said, produced the same yield.

Fries (1948) mentions that thiamine is indispensable for several Basidiomycetes and that biotin is indispensable for some Basidiomycetes.

Melin and Lindeberg (1939) reported that Boletus elegans barely grew in a mineral-dextrose solution containing ammonium tartrate. The addition of thiamine to this basal medium increased growth seven-fold, while the addition of yeast extract increased the growth ten-fold.

Melin and Nyman (1940), using the same basal medium, reported that Boletus luteus and B. variegatus would not grow in this medium unless thiamine was present. They also stated that B. piperatus grew slightly in the medium and that a five-fold increase in growth was observed when thiamine was added. Continuing with their work on boleti, they reported

that B. granulatus grew in the basal medium but addition of thiamine brought about a noticeable increase in growth. B. viscidus would not grow in the basal medium, and the addition of thiamine, although it improved growth, was not adequate. In a later study (1941), they found that certain strains of B. granulatus had an absolute demand for thiamine, and other strains of the same organism, while able to grow without thiamine, are strongly stimulated by it.

Melin and Norkrans (1942) generalized that the mycorrhiza-forming fungi (e.g. boleti) require thiamine for growth. However, one of the mycorrhizal fungi they studied was inhibited by thiamine and its moieties, especially pyrimidine. This organism, they suggested, manufactures enough thiamine for its own use, and any excess is inhibitory.

Treschow (1944) reported that both the white and brown strains of the cultivated mushroom Psalliota bispora* required either thiamine or biotin for growth in synthetic media.

Lindeberg (1941) stated that Marasmius androsaceus would not grow in a synthetic medium containing thiamine unless biotin was added. He confirmed this in a later paper (1944) and also reported that thirteen species of Marasmius tested were heterotrophic for thiamine. In the same paper he reported that some strains of M. perforans had an absolute demand for thiamine while other strains of M. perforans were able to grow without thiamine, but were strongly stimulated by its addition.

*Treschow's terminology for Agaricus campestris.

Leonian and Lilly (1938) found that Collybia tuberosa would not grow on a mineral-dextrose medium containing ammonium nitrate and two per cent agar. Good growth was obtained upon the addition of thiamine or yeast extract to the agar medium. Growth would also occur when amino acids plus thiamine were added to the basal medium.

Marczynski (1943) reported that when thiamine was added to his vitamin-free basal medium, the growth of Collybia velutipes would increase four hundred per cent (figured by dry weight). Biotin, in large doses, was also highly effective, while riboflavine and pyridoxine were ineffective. He also reported that yeast and malt extracts were more active in small amounts (5 mg../26 ml. of medium) than a combination of thiamine, biotin, pyridoxine and riboflavine. He suggested that these results indicated the presence (in the extracts) of a factor or factors other than the vitamins investigated, as yeast extract caused a 7500 per cent increase in dry weight. Marczynski theorizes that the results obtained through the use of relatively large doses of crude products such as yeast extract, malt extract, and Bacto-Peptone suggest the action of assimilable nitrogen. He also points out that small amounts of agar result in an increase in the production of dry matter, possibly through its physical rather than chemical properties.

Lindeberg (1946a) working with three strains of Collybia dryophila showed that they would only grow in synthetic media in the presence of both thiamine and biotin, and that they were absolutely unable to synthesize biotin. In another paper (1946b) he reported that thiamine stimulated the growth of three species of Collybia: 1) ambusta,

2) butyracea, and 3) velutipes. Fifty micrograms of thiamine per liter was all that was necessary to increase the dry weight from 1-2 grams (control flasks) to 30-100 grams.

Hawker (1944), in a review article on the effect which vitamins have upon fungi, reported that, while some Basidiomycetes require thiamine or biotin, none was found to require inositol, pyridoxine, or pantothenic acid. In conclusion, Lilly and Barnett (1951) reported that a fungus may either require vitamins for growth, synthesize them, or else not require them at all.

After reviewing the cited literature, judging from the scope of organisms covered (saprophytes, parasites, and mycorrhizal-forming fungi), it would appear that most Basidiomycetes require one or more B-vitamins.

In all the experiments cited the objective was the fulfillment of nutritional requirements using the weight of mycelium produced as the indicator. In the experiments described here the primary objective was the production of the anti-tumor principle which had been found in sporophores. However, since there seemed to be a logical relationship between the amount of mycelium produced and the elaboration of the active substance, the nutritional experiments, to some extent, paralleled the experiments of others.

Experimental

Forty ml. of basal medium B was placed in each of 63 Erlenmeyer flasks (125 ml. volume). To this was added the amount of nutrilite

listed in Table 3. These flasks were then sterilized in the autoclave at 15 pounds pressure for 15 minutes. While the flasks were cooling, the inoculum of #406f suspension was prepared by blending a quantity of mycelium with sterile distilled water in a sterile (monel metal) Waring Blendor. The density of the inoculum was determined with a Cenco-Photelometer with blue filter. Each flask was inoculated with one ml. of this inoculum and grown for 34 days on the shaker. At the end of this time, the contents of each flask were emptied on tared filter papers, dried for 24 hours at 95°C., and then weighed. The amounts of mutrilites added were chosen on the basis of information obtained in two preliminary experiments. The results appear in Table 3.

A second, more limited experiment was also set up. The procedure was the same, with a few exceptions. The growth-period in this experiment was limited to 14 days. A single flask of each mutrilite studied was inoculated with one ml. of inoculum. However, three inocula of different densities were used. The results appear in Table 4.

For the third experiment in this series, 40 ml. of Lindeberg's solution B (see appendix for formula) was employed as the basal medium. All mutrilites were added to this medium in the amount of 1.0 ml. The growth-period was limited to 15 days. Each flask was inoculated with 1.0 ml. of a blended 406f suspension (22 per cent transmission at 550 mμ). The results of this experiment appear in Table 5.

There were no samples prepared for testing for the presence of an oncostatic principle from the flasks reported in Tables 3, 4, and 5.

Table 3

The effects of nutrilites added to medium B on the growth of Collybia radicata var. furfuracea #406f, shaken for 34 days at room temperature.

Nutrilite*	Amount added to each 40 ml. of medium B	Weight of dried mycelium in mg.
Biotin	10 ml.	240
Biotin	1.0 ml.	310
Biotin	0.1 ml.	300
Calcium pantothenate	10 ml.	220
Calcium pantothenate	1.0 ml.	280
Calcium pantothenate	0.1 ml.	270
Inositol	10 ml.	200
Inositol	1.0 ml.	330
Inositol	0.1 ml.	340
Para-aminobenzoic acid	10 ml.	220
Para-aminobenzoic acid	1.0 ml.	260
Para-aminobenzoic acid	0.1 ml.	320
Aconitic acid	1.0 ml.	290
Aconitic acid	0.1 ml.	270
Aconitic acid	0.01 ml.	270
Adenine	1.0 ml.	260
Adenine	0.1 ml.	240
Adenine	0.01 ml.	410
B_{12}	1.0 ml.	210
B_{12}	0.1 ml.	260
B_{12}	0.01 ml.	240
B_{12}	0.001 ml.	240
Choline chloride	1.0 ml.	290
Choline chloride	0.1 ml.	240
Choline chloride	0.01 ml.	400
Folic acid	1.0 ml.	160
Folic acid	0.1 ml.	160
Folic acid	0.01 ml.	170
Folic acid	0.001 ml.	300

Continued

*Concentration of all nutrilites was 1 mg./ml. except in the case of biotin, folic, acid, and B_{12}, where it was 1 microgram/ml.

Table 3 - Continued

Nutrilite*	Amount added to each 40 ml. of medium B	Weight of dried mycelium in mg.
Nicotinic acid	1.0 ml.	320
Nicotinic acid	0.1 ml.	250
Nicotinic acid	0.01 ml.	230
Nucleic acid	1.0 ml.	330
Nucleic acid	0.1 ml.	290
Nucleic acid	0.01 ml.	310
Nucleinic acid	1.0 ml.	260
Nucleinic acid	0.1 ml.	320
Nucleinic acid	0.01 ml.	270
Pyridoxine HCl	1.0 ml.	460
Pyridoxine HCl	0.1 ml.	360
Pyridoxine HCl	0.01 ml.	360
Ribose	1.0 ml.	310
Ribose	0.1 ml.	370
Ribose	0.01 ml.	360
Thiamine HCl	1.0 ml.	480
Thiamine HCl	0.1 ml.	430
Thiamine HCl	0.01 ml.	420
Adenine triphosphate	0.1 ml.	280
Adenine triphosphate	0.01 ml.	280
Adenine triphosphate	0.001 ml.	300
Desoxyribonucleic acid	0.1 ml.	250
Desoxyribonucleic acid	0.01 ml.	490
Desoxyribonucleic acid	0.001 ml.	330
Riboflavine	0.1 ml.	350
Riboflavine	0.01 ml.	350
Riboflavine	0.001 ml.	280
Controls:		
Basal medium (Medium B)		220
Basal medium		270
Basal medium		280
Basal medium		250
Basal medium		280
Basal medium and 0.5% yeast extract (Medium A)		1010
Basal medium and 0.5% yeast extract		1020

Table 4

The influence of varying inocula and the addition of nutrilites added to medium B on the growth of Collybia radicata var. furfuracea #406f, shaken for 14 days at room temperature.

Nutrilite*	Amount added	Per cent transmission of inoculum	Weight of dried mycelium in mg.
Aconitic acid	1.0 ml.	16	160
Aconitic acid	1.0 ml.	41	200
Aconitic acid	1.0 ml.	55	190
Desoxyribonuleic acid	0.1 ml.	16	240
Desoxyribonuleic acid	0.1 ml.	41	220
Desoxyribonuleic acid	0.1 ml.	55	210
Nucleic acid	1.0 ml.	16	260
Nucleic acid	1.0 ml.	41	230
Nucleic acid	1.0 ml.	55	250
Pyridoxine	1.0 ml.	16	380
Pyridoxine	1.0 ml.	41	210
Pyridoxine	1.0 ml.	55	330
Riboflavine	0.1 ml.	16	400
Riboflavine	0.1 ml.	41	250
Riboflavine	0.1 ml.	55	230
Ribose	1.0 ml.	16	180
Ribose	1.0 ml.	41	210
Ribose	1.0 ml.	55	230
Thiamine	1.0 ml.	16	340
Thiamine	1.0 ml.	41	250
Thiamine	1.0 ml.	55	390
Controls:			
Basal medium (Medium B		16	240
Basal medium		41	210
Basal medium		55	170
Basal medium and 0.5% yeast extract (Medium A)		16	290
Basal medium and 0.5% yeast extract		41	340
Basal medium and 0.5% yeast extract		55	240

*Concentration of all nutrilites was 1 mg./ml.

Table 5

The effects of nutrilites added to Lindeberg's nutrient solution on the growth of Collybia radicata var. furfuracea #406f, shaken for 15 days at room temperature.

Nutrilite*	Weight of dried mycelium in mg.
B_{12}	100
Biotin	100
Folic acid	090
Ribose	110
Nicotinic acid	080
Inositol	090
Nucleic acid	110
Calcium pantothenate	100
Choline chloride	100
Thiamine	100
Riboflavine	100
Pyridoxine	100
Adenine	110
Adenine triphosphate	100
Aconitic acid	090
Para-aminobenzoic acid	080
Desoxyribonucleic acid	090
Nucleinic acid	090
Controls:	
Basal medium (Lindeberg's solution B)	080
Basal medium	110
Medium B	270
Medium A	290

*Concentration of all nutrilites was 1 mg./ml. except in the case of B_{12}, biotin, and folic acid, where it was 1 microgram/ml.

CHAPTER VI

THE EFFECTS OF CARBON SOURCES UPON *COLLYBIA RADICATA VAR. FURFURACEA*

Review of Literature

Carbon, along with nitrogen and inorganic salts, is known to be an essential requirement for life. The role of the carbon source is generally that of supplying energy to the organism. It is also known that species of fungi vary in their ability to utilize different carbon sources for growth and biosynthesis.

Investigations on the availability and utilization of carbon sources by fungi have appeared throughout a period beginning in the late decades of the 19th century and were intensified within the last 25 years. There are many reasons for this latter fact. This kind of investigation has become more fruitful with the appearance of pure chemical compounds, especially the vitamins, whose specific effects could now be recognized and studied.

A far smaller proportion of papers have been written on the use of carbon sources by Basidiomycetes compared to what has been reported for members of the Myxomycetes, Phycomycetes, Ascomycetes, or the Fungi Imperfecti.

Duggar (1905) was the first to attempt to grow cultures of *Agaricus campestris* in media of known composition on gray filter paper to which various carbon compounds were added (along with a mineral nutrient

solution and nitrogen source). He reported that the common sugars, starch, mannite, tartrates, and lactates were unsuccessfully used as carbon sources.

Styer (1928) mentioned that, when A. campestris cultures were grown on a paper medium with mineral nutrients and an ammonium salt, they required the addition of no other carbonaceous material. Both Styer and Duggar concluded that the additional carbon sources were superfluous, and that cellulose played the most important part in the carbon nutrition of this fungus.

However, in a later paper, Styer (1930) showed that A. campestris would not grow in any medium of nutrient salts and soluble organic substances when the total concentration was greater than 0.2M. According to this paper, he grew the mycelium on silica gel plates to which he added various carbon sources by absorption. He obtained the best growth when xylose, glucose, maltose, peach gum, pectin, wheat bran and straw, or granulated peat moss were employed as carbon sources. Cellulose, he reported, failed to support vigorous growth. He theorized that the reason for Duggar's conclusion that sugars failed to support the growth of A. campestris was that the concentration of the culture medium was greater than 0.2M.

Lutz (1925) reported the successful culture of 27 species of Basidiomycetes in a solution of xylose, maltose, ammonium salts and minerals.

Since the writer knows of few publications that appeared after 1935 dealing specifically with the effect of carbon sources on the

nutrition of Basidiomycetes, he had to refer to a great extent to papers dealing with other nutritional aspects of this group of fungi. The data on carbon sources in these papers are more or less incidental.

LaFuze (1937) reported that Polyporus betulinus, Fomes pinicola, and Polystictus versicolor utilized various pentoses, hexoses, and polysaccharides.

Findlay (1941), while investigating the rate of destruction of wood by some wood-rotting fungi, reported the utilization of sucrose and glucose.

Treschow (1944) reported that, while investigating the ability of Psalliota bispora (the commercial strain of what was called A. campestris) to utilize pentoses, hexoses, polyhexoses, salts, and pectins, he obtained the best yields with xylose, apple pectin, calcium malate, arabinose, glucose, calcium oxalate, fructose, galactose, maltose, and sucrose in that order.

Derrick (1949), experimenting with 42 species of wood-destroying Basidiomycetes, reported that she attained maximum growth in media containing 16 per cent glucose.

Khudiakov and Vozniakovskaia (1951) were able to grow four boleti on a medium employing glucose as the carbon source.

Lilly and Barnett (1953) reported on the rate and amount of growth of 57 fungi upon 12 sugars. The following pertinent information on Basidiomycetes appeared: Collybia velutipes utilized fructose, glucose, sucrose, mannose, maltose, and cellobiose best in that order; Polyporus albellus utilized mannose, xylose, glucose, and maltose best in that

order; Polyporus versicolor utilized glucose, mannose, cellobiose, fructose, and maltose best in that order; Lenzites saepiaria utilized maltose, mannose, glucose, and fructose best in that order; Lenzites trabea utilized cellobiose, maltose, glucose, lactose, and mannose best in that order; and Schizophyllum commune utilized glucose, mannose, and fructose best in that order. This information was repeated in the second part of the Handbook of Biological Data (Albritton, Editor, 1953).

Oddoux (1953) reported the successful growth of 247 species of Basidiomycetes (out of 508 species attempted) on a medium using glucose as the sole carbon source.

Regarding ascomycetous fungi, Hawker and Chaudhuri (1946) reported that the mycelial growth of five species increased as the concentration of glucose or fructose increased (up to 10 per cent) in the medium. They also noted that the response of these organisms to more complex carbohydrates was either the same as to glucose and fructose or else of a nature similar to "starvation type growth," i.e., very poor growth. They suggested, these differences in response could be partly explained by the rate at which the particular fungi could break down a complex carbohydrate to hexose.

Some of the information published concerning lower fungi is pertinent and may have a bearing on this investigation.

Czapek (1902, 1902a, 1903), while observing the effects of various nitrogenous sources upon Aspergillus niger, reported that the addition of three per cent sucrose increased the yield of mycelium in all cases except one.

Schade (1940), and Schade and Thimann (1940) reported the unusual finding that Leptomitus lacteus was unable to utilize carbohydrates. This organism used either dl-alanine or l-leucine as its sole source of carbon and nitrogen.

Margolin (1942), in a thesis which appears to be the forerunner of the experiments reported by Lilly and Barnett (1953), grew 22 fungi on common hexoses, mannitol, glycerol, dextrin, and some organic acids. All of the fungi tested were able to grow well on glucose. Twenty of the 22 grew well on fructose. Most of the fungi grew as well on mannose as on glucose.

Van Neil (1944), in a review paper concerning the physiology of microorganisms, stated "A number of experiments have indicated the existence of microorganisms which are able to decompose some polysaccharides but which apparently do not attack the constituent hexose units. A careful study of such cases has, without exception, shown that the interpretation of the experimental results was at fault. One of the most persistent claims, pertaining to the decomposition of cellulose by a group of bacteria unable to utilize glucose, was resolved recently by Stanier who emphasized once again the long known but often forgotten production of toxic products during the heat-sterilization of glucose solutions. There is at present not a single authenticated instance on record of microorganisms which can attack some polysaccharide but not its hydrolytic products."

There are some reports in the literature concerning the preference by organisms of polysaccharides over monosaccharides and disaccharides,

and vice versa.

LaFuze (1937) reported that the wood-rotting fungi which he studied utilized polysaccharides more readily than monosaccharides. Steinberg (1939), in his review paper, suggested that this apparently greater availability might be due to the difficulty encountered in purifying polysaccharides.

Findlay (1941) reported that the wood-rotting fungi he studied utilized glucose more readily than polysaccharides.

Although some of these papers appear contradictory, they may all be correct since it must be kept in mind that each of these investigators studied different organisms. However, any dogmatic statement claiming the existence of organisms that are able to utilize other carbohydrates but not glucose should be studied carefully for misinterpretation of results.

The lower rate of utilization of galactose by many types of organisms has also been reported. Horr (1936) found that Aspergillus niger and Penicillium glaucum 1) produced less mycelium, 2) showed a reduction in spore germination, and 3) showed irregularities in the formation of hyphal threads when galactose was used in the medium instead of glucose. He thought that these effects were not due to the toxic nature of galactose or to its oxidation products but rather to the 1) unavailability, or 2) slow absorption of galactose, which he regarded as a poor source of carbon for fungi.

Edgecombe (1938) reported that five out of the six fungi he studied grew much better in media employing glucose or starch as the carbon source rather than galactose or sucrose.

Margolin (1942) reported that only 12 out of the 22 organisms he studied utilized galactose. In discussing the differences in availability of the four monosaccharides he studied--glucose, fructose, mannose, and galactose--he pointed out that they have the same empirical formula, $C_6H_{12}O_6$, but that there are significant differences in their molecular configurations, which appear below.

H	H	H	H
C = O	H - C - OH	C = O	C = O
H - C - OH	C = O	HO - C - H	H - C - OH
HO - C - H	HO - C - H	HO - C - H	HO - C - H
H - C - OH	H - C - OH	H - C - OH	HO - C - H
H - C - OH	H - C - OH	H - C - OH	H - C - OH
H - C - OH	H - C - OH	H - C - OH	H - C - OH
H	H	H	H
d-glucose	d-fructose	d-mannose	d-galactose

These differences may appear to be only slight. Margolin pointed out, however, that these structural differences gave fructose its ketone quality, and glucose, mannose, and galactose their aldehyde properties. He went on to state that the difference between galactose and glucose is not great, merely a reversal of a hydroxyl group and a hydrogen atom on the fourth carbon atom. Margolin asked "Why then should galactose be so less effective than the other three in supporting the growth of fungi?" He answered his question by stating that the lower four carbon atoms in the molecular configuration of glucose, fructose, and mannose are of the same configuration, while galactose is different,

and that with the exception of galactose, any of the three sugars could be regenerated from either form.

Before closing the discussion of literature concerning carbon sources, a review of papers dealing with the effects of autoclaving should be added.

Lewis (1930) reported that a medium containing four sugars and various nitrogen sources failed to support the growth of Phytomonas malvaceara after being autoclaved for 15 minutes at 15 pounds pressure.

Tanner (1933) stated that, when polysaccharides were sterilized in a steam sterilizer, much more hydrolysis occurred than when an autoclave was employed.

Davis and Rogers (1939) reported that some sugar solutions (especially fructose, glucose, arabinose, and lactose) changed in optical rotation and/or reducing power after being autoclaved for 30 minutes.

Englis and Hanahan (1945) found that the autoclaving of glucose solutions in the presence of a phosphate buffer at a pH of 6.4-6.6 gave a considerable conversion to ketoses, mostly fructose.

Nielsen and Eistrup (1939) and Hartelius and Nielsen (1941) reported the formation of yeast growth substances by heating sugar with organic acids, ammonium tartrate, or ammonium hydroxide.

Since there is the possibility of the tumor-retarding principle, biosynthesized at times by C. radicata var. furfuracea being in part a carbohydrate complex, it should be stated that Derrick (1949) in her thesis reported that the addition of simple sugars (hexoses) to the medium increased the yields of certain polysaccharides as metabolic

products. Werkman and Wilson (1951) however, state that the addition of simple sugars to the medium does not increase polysaccharide products.

Experimental

The discussions of Margolin (1942) and Lilly and Barnett (1953) convinced the writer that his method of evaluating carbon sources should be to supply the same quantity (grams per liter) of carbon in each case. Since the concentration of carbon (excluding the Bacto-peptone) in basal medium B had proved in the past to support luxurious growth of many organisms, this figure was arbitrarily chosen as the standard.

In one liter of medium B, 15 gm. each of glucose and sucrose contain a total of 12.3 gm. carbon. The carbon sources to be tested were made up to contain the same quantity of carbon as existed in medium B.

Thirty-three different carbon sources were investigated. Each carbon source was set up in triplicate as follows: The amount of the carbon source that would yield 4.92 gm. of carbon was weighed and dissolved in 100 ml. of distilled water. Ten ml. of each stock solution was placed in a separate 125-ml. Erlenmeyer flask. Twenty ml. of double-strength medium B (minus the sucrose and glucose it normally contained) was added to each flask. Finally, the volume was brought to 40 ml. by adding 10 ml. of distilled water. In the case of succinic acid this procedure was varied because of its relative insolubility. The amount calculated to yield 4.92 gm. of carbon would not dissolve in 100 ml. of distilled water, but would in 200 ml. Consequently, 20 ml.

of this solution was mixed with 20 ml. of the double-strength medium B (minus the sugars).

After thorough mixing the flasks were plugged and autoclaved for 15 minutes at 15 pounds pressure (121°C.). They were inoculated with one ml. of a suspension (preparation previously described) of 406f (17 per cent transmission at 550 mμ) and placed on the shaker at room temperature (kept at 25°C. by means of an air conditioner). They were harvested after 14 days, and filtered using tared filter papers. The mycelium was dried for 24 hours at 96°C. and the dry weight of the mycelium calculated.

The broth portion, following filtration, was bottled, after addition of 0.01 per cent merthiolate, and shipped to the Division of Experimental Chemotherapy of the Sloan-Kettering Institute for evaluation.

While each sample was being filtered, the pH, color and appearance of the mycelium as well as the medium were recorded.

Three of the carbon sources (honey, ribose, $NaHCO_3$) reported within this first experiment were actually investigated at a later date. However, since the conditions were virtually unchanged, the results are included with this group of data, rather than listed in a separate table. These data appear in Table 6.

Unlike the first experiment which was set up to study the utilization (and subsequent effects) of various carbon sources by C. radicata var. furfuracea, the second experiment was designed to study the effects of carbon sources, incorporated in addition to the main supply of carbon, upon 406f growing in medium B. With this in mind, all of the 33

Table 6

Carbon utilization by *Collybia radicata var. furfuracea* #406f in relation to the tumor-inhibiting properties of *in vitro* cultures, shaken for 14 days at room temperature.

Carbon Source	Weight of dried mycelium in mg.	Weight averaged	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
Monosaccharides								
Pentoses								
d-arabinose	120 100 100	0.11	5.1	5.0	0.6	-	1.04/1.24	+1.5/-1.5
l-arabinose	470 290 420	0.39	6.1	> 5.0	0.8	-	1.28/1.02	0/+0.5
l (+) rhamnose	280 280 290	0.28	7.3	> 5.0	1.0	-	1.11/1.24	+2.0/-1.5
ribose	150 110 160	0.14	5.1	> 5.0	0.8	-	0.99/0.89	-1.5/-1.0
d (+) xylose	510 490 500	0.50	4.8	5.0	0.6	-	0.95/1.04	+2.0/0

*The information in this as well as in the following columns was obtained, in tests of filtrates of the culture media, at the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research.

(continued)

Table 6 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
Hexoses								
d-glucose	440 380 450	0.42	4.8	> 5.0	1.0	±	0.76/1.04	0/0
d-galactose	570 540 540	0.55	5.8	5.0	0.8	-	1.03/1.02	+0.5/+2.0
fructose	240 270 330	0.28	5.1	> 5.0	0.8	-	1.25/1.02	0/+0.5
d (+) mannose	350 400 480	0.41	5.5	> 5.0	0.8	±	0.74/1.04	+1.0/0
l-sorbose	120 120 120	0.12	4.8	> 5.0	0.8	-	0.44/0.26	0/+1.0
Disaccharides								
honey	210 280 240	0.24	5.1	>5.0	0.8	-	0.91/1.13	0/-1.0

(Continued)

Table 6 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
invert sugar	440 550 520	0.50	5.4	5.0	0.6	-	1.33/1.13	+1.0/+0.5
d-lactose	180 170 180	0.18	8.0	> 5.0	0.8	-	1.31/1.02	+1.5/+0.5
maltose	460 470 540	0.49	5.1	> 5.0	0.8	-	0.96/1.04	+0.5/0
sucrose	460 410 660	0.51	5.5	> 5.0	0.8	-	0.87/1.04	+0.5/0
Trisaccharides								
d (+) melezitose	590 450 710	0.58	5.6	5.0	0.8	-	0.92/0.89	0/+0.5
raffinose	280 400 400	0.36	4.8	> 5.0	0.8	-	1.06/1.16	0/0

(Continued)

Table 6 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
Polysaccharides								
dextrin	370 180 150	0.23	4.8	> 5.0	0.8	-	1.17/1.02	+0.5/+0.5
Polyhydric alcohols								
glycerol	140 150 130	0.14	7.9	> 5.0	0.8	-	1.23/1.02	+1.5/+0.5
d-mannitol	170 260 190	0.21	7.4	> 5.0	0.8-0.4	-	1.29/1.02	-0.5/+0.5
sorbitol	190 180 190	0.19	7.8	5.0	0.6	-	1.11/1.24	+3.5/-1.5
Organic acids								
citric acid	140 170 160	0.16	2.0	0.5	0.5 (dil 1-10)	-	0.96/1.24	-0.5/-1.5

(Continued)

Table 6 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
glycolic acid	080 070 070	0.07	2.3	0.5	0.5(dil 1-10)	-	1.20/1.08	-1.5/+0.5
levulinic acid	060 070 070	0.07	3.3	0.5	0.5(dil 1-10)	-	1.16/1.24	-0.5/-1.5
dl-malic acid	090 070 080	0.08	2.3	0.5	0.5(dil 1-10)	-	1.00/1.24	-1.0/-1.5
l-malic acid	140 140 150	0.14	2.0	0.5	0.5(dil 1-10)	-	1.28/1.24	-1.0/-1.5
malonic acid	070 050 060	0.06	1.6	0.5	0.5(dil 1-10)	-	1.12/1.08	-1.0/+0.5
propionic acid	020 020 030	0.02	3.3	0.5	0.5(dil 1-10)	-	1.12/1.08	0/+0.5
pyrogallic acid	060 070 100	0.08	4.0	3.0(dil 1-10)	0.3(dil 1-10)	-	1.38/1.02	+0.5/+0.5

(Continued)

Table 6 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
pyroligneous acid	020 020 030	0.02	3.9	5.0	0.6	-	1.01/1.24	0/-1.5
succinic acid	070 060 070	0.07	2.3	0.5	0.5(dil 1-10)	-	1.21/1.13	0/+0.5
d-tartaric acid	110 110 110	0.11	2.0	0.5	0.5(dil 1-10)	-	1.23/1.24	-1.0/-1.5
Inorganic salts								
$NaHCO_3$	160 170 180	0.17	10.5	0.5 RETEST:	0.5(dil 1-10) 0.5(dil 1-5)	- -	1.26/1.24 1.06/1.02	-0.5/+1.5 +1.0/0
Growth controls								
basal medium B	780 880 830	0.83	5.4					
basal medium B	600 670 640	0.64	5.8					

previously used carbon sources, as well as seven other compounds, were investigated. Five ml. of each of the 33 stock solutions (preparation previously described) except ribose was placed (in triplicate) in separate 125-ml. Erlenmeyer flasks. Twenty ml. of double-strength medium B was added to each flask. Finally, the volume was brought to 40 ml. by adding 15 ml. of distilled water.

In the case of succinic acid, 10 ml. of the stock solution was added to 20 ml. of double-strength medium B and made up to 40 ml. by adding 10 ml. of distilled water.

The seven new carbon sources investigated (and ribose) were divided into two groups.

Group I included n-acetyl glucosamine, glucosaminic acid, d+ glucosamine-HCl, trehalose, and ribose. These were present in the amount of 0.333 gm. per flask. One gram of each compound was weighed and dissolved in 60 ml. of double-strength medium B. Twenty ml. of this solution was placed into each of three 125-ml. Erlenmeyer flasks, and 20 ml. of distilled water was added.

Group II included inulin, uric acid, and alpha-d-glucose pentaacetate. Because of their relative insolubility, 0.01 gram of these compounds was dissolved in 60 ml. of double-strength medium B and 60 ml. of distilled water. This total (120 ml.) was then divided into three 40 ml. aliquots which were put respectively into three 125-ml. Erlenmeyer flasks.

After thorough mixing, the pH, color, and the appearance of the medium in each flask were determined. The flasks were then plugged

and autoclaved for 15 minutes at 15 pounds pressure (121°C.). Each flask was inoculated with one ml. of a suspension (preparation previously described) of 406f (21 per cent transmission at 550 mμ) and placed on the shaker at room temperature (kept at 25°C.).

The flasks were removed after 17 days, and the contents filtered using tared filter paper. The mycelium was dried for 24 hours at 95°C. and the dry weight of the mycelium was calculated.

Eighty ml. of the broth portion of each flask, following filtration, was methiolated, bottled, and shipped to the Sloan-Kettering Institute to be tested for the presence of a tumor-retarding principle.

While each sample was being filtered, the pH, color and appearance of the mycelium as well as the medium were recorded.

The data pertinent to carbon experiment 2 are to be found in Table 7.

Table 6 shows that, when mannose and glucose were employed as the carbon source in medium B, C. radicata var. furfuracea biosynthesized a tumor-retarding principle. Confirmation of these findings was essential.

The experiment was modified quantitatively by increasing the total volume threefold (to 120 ml.), and also by changing the vessel (to a 500-ml. Florence flask).

Four flasks, two containing mannose and two containing glucose, were inoculated with a 406f suspension, and placed on the shaker for 19 days at room temperature. At the end of this period, eight samples were prepared from the four flasks in the following manner:

Table 7

Effects of addition of carbon sources to medium B upon Collybia radicata var. furfuracea #406f in relation to the tumor-inhibiting properties of in vitro cultures, shaken for 17 days at room temperature.

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam of control tumor	Wt. change in gm./wt. change of control
Monosaccharides									
Pentoses									
d-arabinose	440 410 350	0.40	5.3	4.3	5.0	0.8	-	0.93/1.05	0/-1.5
l-arabinose	300 380 370	0.35	5.3	5.0	> 5.0	0.8	-	1.19/1.24	0/+1.5
l (+) rhamnose	370 380 380	0.38	5.3	5.0	> 5.0	0.8	-	1.32/1.24	0/+1.5
ribose	400 380 300	0.36	5.3	4.0	> 5.0	0.8	-	0.91/1.12	-0.5/+1.5
d (+) xylose	440 350 460	0.42	5.3	5.1	> 5.0	0.8	-	1.04/0.91	+1.5/0

*The information in this as well as in the following columns was obtained, in tests of filtrates of the culture media, at the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research.

(continued)

Table 7 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
Hexoses									
d (+) glucose	350 550 340	0.45	5.3	4.4	> 5.0	0.8	-	1.00/1.03	+0.5/+1.0
d-galactose	450 450 490	0.46	5.3	5.1	> 5.0	0.8	±	0.81/1.13	-1.5/-1.0
fructose	440 860 640	0.65	5.3	4.9	> 5.0	0.8	-	1.18/1.24	0/+1.5
d (+) mannose	410 350 300	0.35	5.4	5.1	> 5.0	0.8	-	0.82/1.03	-1.0/+1.0
l-sorbose	480 290 380	0.38	5.3	3.9	> 5.0	0.8	-	0.90/1.06	-1.5/0
Others									
n-acetyl-glucosamine	180 200 150	0.18	5.3	5.3	> 5.0	0.8	-	1.14/1.06	-1.0/-1.0

(continued)

Table 7 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
d (+) glucosamine-HCl	200 190 210	0.20	5.3	3.4	> 5.0	0.8	-	1.02/1.06	+0.5/-1.0
glucosaminic acid	270 260 320	0.28	5.3	4.3	> 5.0	0.8	-	0.98/1.05	+2.0/-1.5
alpha-d-glucose-pentaacetate	280 390 370	0.35	5.3	5.0	> 5.0	0.8	-	0.90/1.12	+2.0/+1.5
Disaccharides									
honey	370 360 380	0.37	5.3	5.3	5.0	0.6	-	0.96/1.13	-0.5/-1.0
invert sugar	790 820 700	0.77	5.3	5.0	5.0	0.6	-	0.89/1.13	-1.5/-1.0
d-lactose	570 450 570	0.53	5.3	5.0	>5.0	0.8	-	1.23/1.24	+0.5/+1.5
maltose	330 370 370	0.36	5.2	5.2	>5.0	0.8	-	0.93/1.13	-0.5/-1.0

(continued)

Table 7 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
sucrose	710 660 580	0.65	5.3	3.9	5.0	0.8	-	0.87/0.91	+1.5/0
trehalose	390 280 300	0.32	5.3	5.2	5.0	0.6	-	0.98/1.06	+1.0/-1.0
Trisaccharides									
d (+) melezitose	340 340 340	0.34	5.3	5.3	5.0	0.6	-	0.99/1.05	0/-1.5
raffinose	320 250 310	0.29	5.4	5.1	> 5.0	0.8	-	0.87/1.06	-1.0/-1.0
Polysaccharides									
dextrin	460 380 450	0.43	5.3	4.5	> 5.0	0.8	-	1.19/1.24	-0.5/+1.5
inulin	220 330 Contaminated	0.28	5.3	4.3	5.0	0.6	-	0.99/1.13	+0.5/-1.0

(continued)

Table 7 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
Polyhydric alcohols									
glycerol	320 420 Contaminated	0.37	5.4	5.0	> 5.0	0.8	-	1.19/1.24	+0.5/+1.5
d-mannitol	250 270 290	0.27	5.4	5.4	> 5.0	0.8	-	1.20/1.24	-1.0/+1.5
sorbitol	310 290 270	0.29	5.3	5.2	5.0	0.6	-	1.32/1.24	0/+1.5
Organic acids									
citric acid	150 170 160	0.16	1.9	2.0	0.5 RETEST:	0.6(dil 1-10) 0.5(dil 1-5)	- -	0.93/1.06 1.04/1.02	0/0 0/0
glycolic acid	080 070 070	0.07	2.2	2.0	0.5 RETEST:	0.5(dil 1-10) 0.5(dil 1-5)	- -	0.93/0.89 1.18/1.45	0/-1.0 0/-0.5
levulinic acid	100 120 140	0.12	2.9	2.9	1.0	0.5(dil 1-2.5)	-	0.87/0.89	-0.5/-1.0

(continued)

Table 7 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
dl-malic acid	120 110 120	0.12	2.2	2.4	1.0	0.5(dil 1-2.5)	-	0.93/0.89	-1.5/-1.0
l-malic acid	150 120 150	0.14	1.9	2.2	0.5	0.5(dil 1-10)	-	0.93/0.89	0/-1.0
malonic acid	110 100 090	0.10	1.6	1.6	0.5 RETEST:	0.5(dil 1-10) 0.5(dil 1-5)	- ±	0.97/0.89 1.03/1.45	-0.5/-1.0 -2.0/-0.5
propionic acid	130 120 120	0.12	2.7	2.9	1.0	0.5(dil 1-2.5)	-	1.10/1.14	-2.0/0
pyrogallic acid	180 180 180	0.18	5.3	4.9	0.5 RETEST:	0.5(dil 1-10) 0.5(dil 1-5)	- -	0.89/1.06 1.07/1.02	-0.5/0 0/0
pyroligneous acid	110 110 100	0.11	4.0	4.0	5.0	0.8	-	0.84/1.05	-0.5/-1.5
succinic acid	140 120 130	0.13	2.8	2.6	1.0	0.5(dil 1-5)	-	1.36/1.14	-2.5/0
d-tartaric acid	140 140 140	0.14	2.0	2.0	0.5 RETEST:	0.5(dil 1-10) 0.5(dil 1-5)	- -	1.18/1.14 1.25/1.45	-1.0/0 -1.0/-0.5

(continued)

Table 7 - Continued

Carbon source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
uric acid	360 350 370	0.36	5.3	5.1	> 5.0	0.8	-	1.00/1.06	+1.0/-1.0
Inorganic salts									
$NaHCO_3$	140 130 160	0.14	8.2	10.4	1.0 RETEST:	0.5(dil 1-5) 0.5(dil 1-2.5)	- ±	1.23/1.14 1.00/1.45	-1.5/0 -2.0/-0.5
Growth Controls									
basal medium B	250 240 240	0.24	5.3	5.3					
basal medium A	920 960 070	0.98	5.6	5.6					

The contents of the two flasks containing mannose were mixed and the culture broth separated from the mycelium by filtering through Sargent filter paper #500. Two samples each were prepared from the mycelium and the broth. An 85-ml. aliquant of the broth was bottled and merthiolate added. Similarly, an 85-ml. aliquant was bottled after it had been rendered sterile by filtering through a Seitz-filter pad (Hercules Filter Corp. type ST).

The mycelium obtained from the two flasks was extracted by blending it in a Waring Blendor for two minutes with distilled water (1:1), and filtered through Sargent paper #500. An 85-ml. aliquant of this extract was bottled, and merthiolate was added. Similarly, an 85-ml. aliquant of this extract was bottled after it had been rendered sterile by filtering it through a Seitz-filter pad.

Four samples were similarly produced from the contents of the two flasks containing glucose. The two methods of preservation were employed to determine what differences, if any, would occur in the evaluating tests conducted by the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research.

Neither the weights of the mycelia, nor changes in color, appearance, or pH were recorded; the biosynthesis of the tumor-retarding principle was the only criterion of interest.

A larger experiment, with further variations was conducted subsequently. In this case, 406f was grown in one liter of medium in Fernbach flasks (capacity 2.8 liters) for 23 days on the shaker at room temperature. The media employed were "A" and "B" with 1, 3, 5, and 7

per cent glucose substituted in place of the usual sugars. Eight samples were prepared by extracting the mycelium with the culture broth it grew in, bottling an 85 ml. aliquant, and adding merthiolate.

An 85 ml. aliquant of each of the extracts prepared using medium "A" was mixed with 85 ml. of absolute ethanol, and placed in the refrigerator for one hour. The precipitate that formed was centrifuged, and dissolved in 85 ml. of distilled water, bottled, and merthiolate was added.

The mycelial mat (of the flasks containing medium B) which remained on the filter paper following the extraction process was blended with absolute ethanol (1:1), placed in large evaporating dishes and allowed to evaporate to near dryness. Distilled water was added to bring it to the original volume, the mixture blended in a Waring Blendor, filtered, bottled, and merthiolated.

The remaining portion of the eight extracts was frozen and stored in that state. Upon thawing, it was observed that there were two distinct fractions in each beaker: 1) a mucilaginous portion, and 2) a liquid portion. Samples of each of these fractions were prepared by bottling 85 ml. aliquants, and adding merthiolate.

All of the preparations described up to this point, with one exception, were negative according to the evaluation of the Sloan-Kettering Institute. The mycelial extract of 406f grown in mannose (first series described) was given a $\pm$ rating. This result is reported in Table 8.

Table 8

Mannose utilization by *Collybia radicata var. furfuracea* #406f in relation to the tumor-inhibiting properties of *in vitro* cultures, shaken for 19 days at room temperature.

Concentration of mannose	Description of sample	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
3.69 gm./120 ml.	mycelium, blended with water 1:1, merthiolate added	5.0	0.6	±	0.77/1.12	+1.5/+1.5

*

*The information in this as well as in the following columns was obtained at the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research.

Additional experiments were performed with mannose as the substituted sugar. The conditions of the first experiment were identical with those described for the second glucose experiment with the following exceptions: 1) the concentrations used were 1, 4, and 8 per cent mannose, and $1^1/_2$ per cent each of mannose, glucose, and galactose; 2) the growth period was 27 days.

Eight samples were prepared by extracting the mycelium with the culture broth it grew in, filtering, bottling an 85 ml. aliquant, and adding merthiolate. The remaining portion of the eight extracts was frozen and stored in that state. Samples were prepared from the mucilaginous portions and the liquid portions after thawing the 1, 4, and 8 per cent mannose in medium A extracts.

One final experiment concerning the effects carbon sources had upon Collybia radicata var. furfuracea was performed. A suspension of 406f was used to inoculate ten 500-ml. Florence flasks containing 125 ml. of medium A. The cultures were grown on the shaker for 21 days at room temperature. At this time, the remaining culture broths were decanted, and replaced with sterile 125-ml. quantities of three per cent mannose, three per cent glucose, three per cent galactose, one-half of one per cent glucosamine hydrochloride, and one-tenth of one per cent ribose solutions respectively (duplicate flasks of each carbon source were made). These flasks were again placed on the shaker. One set was removed after 12 days, and the second set after 26 days. An extract was prepared from each flask by blending the mycelium in a Waring Blendor with the carbonaceous solution it grew in. This extract was then filtered and bottled, and merthiolate was added.

All of the preparations in this second series of experiments, with two exceptions, were negative according to the evaluation of the Sloan-Kettering Institute for Cancer Research. The extracts prepared from 406f mycelium grown in Fernbach flasks in the one and the eight per cent mannose (substituted in one liter of medium A) concentrations were given a ± rating. These data are reported in Table 9.

Certain preparations of the first two carbon experiments gave negative results according to the system of tumor evaluation of the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research. However, the consistent depression in tumor growth observed, when some constituents were incorporated into the culture medium, prompted a statistical analysis of the results. The F test from an analysis of variance performed on observations made on the individual mouse tumors (data supplied by the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research) disclosed significant depression of mouse sarcoma 180 by filtrates of the media when either citric acid or honey served as the sole carbon source (Table 6), and by filtrates of the media when mannose and invert sugar served as additional carbon sources (Table 7). When subsequent experiments with carbon sources (see pages 67-71) showed that previously active materials gave negative results according to the Sloan-Kettering evaluation system, it was found to be of interest to subject the results to statistical analysis. Two of the preparations, the filtrates containing glucose and mannose as sole carbon sources, were shown to depress significantly the growth of mouse sarcoma 180 (Table 10).

Table 9

Mannose utilization by *Collybia radicata var. furfuracea* #406f in relation to the tumor-inhibiting properties of *in vitro* cultures, shaken for 27 days at room temperature.

Concentration of mannose	Description of sample	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
1%	mycelium blended with broth, merthiolate added	3.0	0.5-0.4	±	0.84/1.24	-3.0/-1.0
4%	mycelium blended with broth, merthiolate added	3.0	0.3-0.2	-	1.04/1.24	-2.0/-1.0
8%	mycelium blended with broth, merthiolate added	> 5.0	0.8-0.4	±	0.84/1.24	-3.0/-1.0

*The information in this as well as in the following columns was obtained at the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research.

Table 10

Significant depression of mouse sarcoma 180 after treatment with filtrates of Collybia radicata var. furfuracea #406f.

Carbon Sources in Medium	Tumor size expressed as per cent of control tumor
Citric acid, 3%	77*
Honey, 3%	80*
Mannose, 3%	76*
Glucose, 3%	82*
Additives to Medium B (see Appendix)	
Mannose, 1.5%	81*
Invert sugar, 1.5%	78**

* 0.05 level of P

** 0.01 level of P

CHAPTER VII

SUGAR DETERMINATION

Review of Literature

Because of the correlation between the biosynthesis of the tumor-retarding principle and the presence of certain sugars in the culture medium, it appeared to be of interest to know the sugar content of C. radicata var. furfuracea mycelium produced in vitro.

The possibility was considered that a supply of a simple nutrient like glucose would enable the fungus to synthesize the active material at an accelerated rate.

The chemical composition of fungi has been previously reported. Gussow and Odell (1927) cite the following information as the composition of Agaricus campestris:

Water	93-95%
Nitrogen substances	7-8%
Non-nitrogen substances	4%
Small amounts of nutritive salts and sugars.	

The Handbook of Chemistry and Physics (Hodgman, 1950) lists the following data for "mushrooms" under "Composition and value of foods":

Protein	3.5% by weight
Fat	0.4% by weight
Carbohydrates	6.0% by weight

The Handbook of Biological Data, (2nd Part, Standard Values in Nutrition and Metabolism, Albritton, Editor, 1953) lists the following

data for mushroom (Agaricus compestris):

Energy value	16 calories/100 g.
Water	91 g./100 g.
Protein	2.4 g./100 g.
Fat	0.3 g./100 g.
Total carbohydrate	4.0 g./100 g.
Crude fiber	0.9 g./100 g.
Ash	1.1 g./100 g.

Because of discrepancies in the composition figures, and also to serve for comparison, the total sugar content of the commercial mushroom, A. campestris was first determined. The Michigan Mushroom Company, Niles, Michigan, supplied us with the fresh sporophores used for these experiments.

The first attempts at determining the total sugar content (normal acid hydrolysis, boiled for 10 minutes) seemed to indicate that this fungus contained no sugar.

Bourquelot and Herissey (1904 and 1905) established the presence of trehalose (generally) and cane sugar (sometimes) in mushrooms. They reported that Boletus edulis, B. aurantiacus, and Cortinarius elatior contained one per cent trehalose; B. badius and Amanita muscaria two per cent trehalose; and Russula delica, R. Queletii, and Paxillus involutus three per cent trehalose.

Iwanoff (1925) showed that the sugar contained in most fungi is trehalose, and stated that it could be split only with great effort. He suggested that for complete hydrolysis, the material should be boiled for six hours with five per cent sulphuric acid.

Davis and Rogers (1939) reported trehalose to be one of those sugars that belong to the stable group; i.e., one not affected by autoclaving for 30 minutes at 120°C.

Experiments to determine what method of hydrolysis would be most suitable were made. One method, using the enzyme trehalase, was considered. Bourquelot and Herissey (1904) reported finding trehalase present in the caps of Boletus edulis, B. aurantiacus, B. badius, and Cortinarius elatior. Iwanoff (1925) stated that trehalase, though present in most fungi, varies in concentration in different parts of the fruiting body and also sometimes is not present, and then appears in later stages of growth.

Myrbäck and Örtenblad (1936, 1937a and 1937b) while describing the isolation of this enzyme from yeast reported that one gram of trehalose dissolved in 10 ml. of the crude enzyme preparation was 57 per cent hydrolyzed at the end of 47 hours. The amount of work this entailed, and the relatively low yield obtained during the long period of hydrolysis prompted the search for an easier and more accurate method. This method used acid hydrolysis with and without pressure.*

Experimental

Twenty-five mg. of trehalose hydrate was added to each of twelve 100-ml. volumetric flasks containing 50 ml. of distilled water. Two ml. of concentrated hydrochloric acid was added to each flask. Six of these flasks were then placed in a bath of boiling water. Two flasks were removed at the end of 90, 180, and 360 minutes. The remaining six

*Outline and supervision of this experiment by Dr. E. Benne and technical help extended by Mr. W. S. Brammell, both of the Michigan Agricultural Experiment Station, are gratefully acknowledged.

flasks were placed in an autoclave (15 pounds pressure, 121°C.) and two flasks each were removed at the end of 30, 60, and 90 minutes. When all 12 flasks were cool they were made nearly neutral with sodium hydroxide, and distilled water added until a volume of 100 ml. was reached. The solution was then filtered through Whatman filter paper #42. A 50-ml. aliquot was transferred into a 400-ml. beaker containing 25 ml. of a stock copper sulfate solution and 25 ml. of alkaline tartrate solution. The contents of the beaker were covered with a watch glass and heated on asbestos gauze over a Bunsen burner. The flame was adjusted so that the contents began to boil in four minutes, and the boiling allowed to proceed for exactly two minutes. The hot solution was immediately filtered through an asbestos mat in a porcelain Selas crucible using suction. The precipitated cuprous oxide was washed thoroughly with hot water, and then dried for two hours at 105°C. The Selas crucibles were removed, placed in a desiccator, and were weighed after sufficient cooling. A blank determination was also performed.

Since trehalose hydrate (Mol. wt. 378.34) was used, the material actually contains only 90.48 per cent of anhydrous trehalose (Mol. wt. 342.31). With this correction in mind, therefore, each volumetric flask is observed to contain 22.62 mg. of anhydrous trehalose. Table 11 shows the results of this experiment.

These data indicate that the most efficient method of hydrolyzing trehalose is that which applies pressure in an autoclave for 30 minutes. The time required in this case is 1/12 of that needed to obtain comparable results with acid hydrolysis in a bath of boiling water.

Table 11

Hydrolysis of Trehalose
(sugar determined as glucose)

Boiled	Sample number	Grams	Per cent	Per cent averaged
90 minutes	1	0.01341	56.32	57.4
90 minutes	2	0.01394	58.55	
180 minutes	3	0.01870	78.54	80.2
180 minutes	4	0.01947	81.77	
360 minutes	5	0.02284	95.93	93.5
360 minutes	6	0.02166	90.97	
Autoclaved				
30 minutes	7	0.02280	95.76	98.1
30 minutes	8	0.02390	100.38	
60 minutes	9	0.02288	96.10	97.4
60 minutes	10	0.02350	98.70	
90 minutes	11	0.02332	97.94	96.1
90 minutes	12	0.02244	94.25	

Three samples of mushrooms were analyzed for total sugar using the method described. The following kinds of mushroom tissue were divided into analytical portions:

a) slices of context, i.e., the spongy tissue in the cap excluding the gills, of A. campestris.

b) slices of whole mushroom (A. campestris).

c) in vitro mycelium of C. radicata var. furfuracea (washed three times with distilled water).

Fifty gm. of each material was put into a Waring Blendor and blended for four minutes with 100 ml. of distilled water. The slurry was

transferred to a 250-ml. volumetric flask containing two ml. of saturated lead acetate solution (to precipitate the protein present), mixed thoroughly, and allowed to stand for at least 15 minutes.

Approximately two gm. of solid potassium oxalate (used to precipitate the excess lead present) was added to each flask, and sufficient water added to bring the volume up to 250 ml. The contents were mixed thoroughly to dissolve the salt and allowed to stand at least 30 minutes.

A portion of the extract was filtered into an Erlenmeyer flask through S. & S. #588, 12.5 cm. folded filter paper. A 50-ml. aliquant was pipetted into a 100-ml. volumetric flask, two ml. of concentrated hydrochloric acid was added, and hydrolyzed in an autoclave for 30 minutes at 15 pounds pressure (121°C.).

After the flasks were removed from the autoclave and permitted to cool, they were made nearly neutral with sodium hydroxide solution, the volume was brought to 100 ml. using distilled water, and the solution mixed. The contents were filtered through Whatman filter paper #42 and a 50-ml. aliquot was transferred to a 400-ml. beaker containing Fehling's solution. The evaluation of reducing sugars was then performed as previously described. A blank determination was also made and subtracted. Corrected figures appear in Table 12.

The solid and moisture content of A. campestris was determined by weighing four samples of the fresh whole mushroom, drying them for 24 hours at 99°C. and reweighing the samples. The resulting calculations are seen in Table 13.

Table 12

Comparison of sugar content determined for *A. campestris* with that of *in vitro* mycelium of *C. radicata var. furfuracea* #406f.

Sample	Number	Grams	Glucose determined: Per cent	Glucose determined: Per cent averaged
A. campestris, context	1	0.01274	0.255	0.252
context	2	0.01240	0.248	
A. campestris, whole mushroom	3	0.03081	0.616	0.608
whole mushroom	4	0.03000	0.600	
C. radicata, var. furfuracea, *in vitro* mat	5	0.03351	0.670	0.682
	6	0.03467	0.693	

Table 13

Moisture determination of *A. campestris*

Sample number	Weight of fresh mushroom	Weight of dried mushroom	Per cent solids	Per cent moisture
1	4.6975	0.3637	7.74	92.26
2	9.7808	0.6128	6.26	93.74
3	15.0447	1.5025	9.98	90.02
4	31.9511	2.3330	7.30	92.70
		Average	7.82	92.18

CHAPTER VIII

THE EFFECT OF NITROGEN SOURCES

Review of Literature

Robbins (1937), following a survey he made of the literature up to that time, suggested that all organisms could be classified into four groupings on the basis of their nitrogen requirements: Group I to contain the nitrogen-fixing organisms, which could also assimilate nitrate, ammonium, and organic nitrogen; Group II to contain the nitrate-ammonium users--those unable to utilize gaseous nitrogen but living on nitrate nitrogen and also able to assimilate ammonium and organic sources of nitrogen; Group III, the ammonium users, utilizing only ammonium and organic nitrogen sources; and Group IV, the organic-nitrogen users, which could grow only when nitrogen was supplied in a complex form.

The above reference, as well as many of those which follow, was removed from the normal chronological sequence to give greater lucidity to this review. In general, chronological order is maintained only insofar as the various individual genera or natural groupings are concerned.

Czapek (1902, 1902a, and 1903) studied the effects of various inorganic and organic nitrogen sources upon the growth of Aspergillus niger with and without three per cent sucrose added (and a solution of mineral salts). He stated that, while A. niger could utilize nitrates

and ammonium salts, mycelial proteins are most easily synthesized from amino acids and those substances which most nearly resemble the amino acids. For example, he explained the high utility of acetamid as a nitrogen source by the fact that its structure approaches that of an amino acid. This work is generally recognized as a pioneering contribution in the nitrogen nutrition of the fungi.

Klotz (1923) reported that amino nitrogen was most readily assimilated by the fungi he studied. He concluded that the factors influencing the nitrogen content of the fungous mat are the nitrogen and carbon sources of the medium, the length of incubation, the rate of growth, and the hydrogen ion concentration.

Duggar (1905) is credited with being the first to culture successfully A. campestris in vitro. He reported the organism indicated a preference for proteins, ammonium salts, and nitrates in this order.

Styer (1928) reported that ammonium salts, urea, glycine, asparagine, peptone, and proteins were all good nitrogen sources for A. campestris, although the more complex forms gave a slightly denser growth than did the inorganic nitrogen compounds. Later (1930), following extensive subsequent experiments, he substantiated his original findings by declaring ammonium nitrate, leucine, nucleic acid, nucleoprotein, glutenin, casein, and albumin to be the better nitrogen sources for this organism.

Treschow (1944) found A. campestris unable to assimilate nitrate nitrogen while able to utilize ammonium nitrogen, especially the more complex salts. He added that amino acids were the best source of

nitrogen, especially asparagine and glutamic acid. However, he found that when both ammonium salts and amino acids are present in the medium at the same time, the fungus was unable to select freely the more suitable source of nitrogen.

Humfeld (1948) grew A. campestris in submerged culture in media incorporating either asparagus butt juice or press juice from pear water as the main substrate.

Humfeld and Sugihara (1949) reported that this organism could utilize ammonium, urea, amino acids, and some of the more complex forms of organic nitrogen.

Later (1952), while studying the nutritional requirements of A. campestris, they used a medium employing urea as the nitrogen source. They reported they chose urea "because its utilization does not change the pH of the medium sufficiently to have an unfavorable effect on growth. Also, when utilized, it does not leave any residual radical in solution."

Hawker (1936) reported that she grew Collybia velutipes on a mineral-dextrose medium containing potassium nitrate.

Leonian and Lilly (1938) stated that C. tuberosa did not grow on a mineral-dextrose medium containing ammonium nitrate unless amino acids and thiamine were added. This work illustrates well that some of the earlier reports of organisms being unable to utilize some forms of nitrogenous compounds (as compared to yeast and malt extracts) may have been due to the species' need for vitamins which were unknown at the time the investigations were performed.

Lindeberg (1946b) reported that C. ambusta, C. butyracea, and C. velutipes grew on a medium employing ammonium tartrate as the nitrogen source. Robbins (1950) reported that only one of two Collybia sp. grew well on a medium containing asparagine as its nitrogen source. Norkrans (1950), while studying 17 strains of nine Tricholoma sp., found that ammonium and organic nitrogen compounds could be assimilated by all species, but only T. nudum could utilize nitrate nitrogen. Later (1953) she confirmed these findings by using an ammonium salt as the nitrogen source in a basal medium which supported the growth of seven Tricholoma sp. Oddoux (1953) reported the growth of 247 species of Basidiomycetes (out of 508 species attempted) on a medium whose chief nitrogen source was ammonium chloride.

Melin and Lindeberg (1939) reported that they grew Boletus elegans in a medium containing ammonium tartrate as its nitrogen source. How (1940) confirmed the ability of B. elegans to utilize ammonium nitrogen by growing it upon a medium containing ammonium chloride as the nitrogen source. Melin and Nyman (1940) grew B. granulatus, B. luteus, B. variegatus, B. piperatus, and B. viscidus in a medium employing ammonium tartrate as its nitrogen source. The last mentioned species, however, did not grow as well as the other four. Khudiakov and Vozniakovskaia (1951) reported the growth of B. luteus, B. variegatus, B. luridus, and B. edulis on a medium containing ammonium nitrate, tryptophane, and casein hydrolysate among other nutrients. They concluded that these four species were aminoheterotrophic. Melin and Nilsson (1952) proved that B. variegatus in mycorrhizal connection with trees

could assimilate ammonium nitrogen by using a labelled (N^{15}) salts and later isolating it from the root tissues, stem, and needles of a pine seedling. Lutz (1925) grew 27 species of Basidiomycetes in a solution containing two ammonium salts as the nitrogen source.

La Fuze (1937) while studying the nutritional characteristics of Polyporus betulinus, Fomes pinicola, and Polystictus versicolor found that amino and ammonia nitrogen was better utilized than amide, nitrate, and nitrite nitrogen. He also observed that 1) the best growth occurred on proteins containing relatively large amounts of glutamic acid and tryptophane, and 2) the amino acids which contained phenyl and disulphide radicals retarded growth.

Leonian and Lilly (1938) reported that generally, of 25 organisms tested, growth improved when amino acids were substituted for ammonium nitrate in the medium. Among the amino acids used, l-aspartic acid and d-glutamic acid were utilized best by Coprinus lagopus and Pleurotus corticatus, while Collybia tuberosa utilized d-arginine and l-aspartic acid best.

Lindeberg (1941) grew Marasmius androsaceus on a medium employing ammonium tratrate as the nitrogen source. Later (1944) he stated that only M. fulvobulbillosus out of 13 species of Marasmius that he studied could utilize nitrate nitrogen (KNO_3). All 13 species utilized ammonium chloride and asparagine, the latter better than the former. These two nitrogen sources were superior to alanine, leucine and urea which were good sources for all species; glycine was utilized by only one species and acetamide by two. He theorized that asparagine was better than

ammonium salts because the NH_3 escapes from the medium leaving the Cl^- ion, which unites with H^+ ions to produce an unfavorable environment for the fungi.

Derrick (1949) while studying the nutrition and physiology of 42 wood-destroying Basidiomycetes found that, while none of the organisms required organic nitrogen, they generally grew better in organic nitrogen sources than in ammonium salts. She concluded that, as previously reported by La Fuze (above) and others, as the nitrogen compounds became simpler, the resulting growth was less. She also reported that the organisms she studied did not utilize either nitrates or nitrites, or ammonium chloride in the absence of succinic acid. In general, she stated, maximum growth and maximum rate of growth was attained in malt extract medium rather than in the synthetic medium she used, even though the nitrogen content of the latter was three times as large.

In a later report of this work, Jennison, Newcomb, and Henderson (1955) further elaborated on the growth-supporting ability of amino acids compared with casein hydrolysate to be as follows for 15 species of wood-rot fungi: Casein hydrolysate, DL-valine, glutamine>DL-aspartic acid, L-glutamic acid, L-arginine, L-asparagine, glycine, L-proline, DL-alpha alanine, DL-ornithine>L-hydroxyproline, L-tryptophane, DL-serine, DL-threonine, L-leucine, DL-phenylalanine, DL-methionine, DL-isoleucine>L-histidine, DL-norleucine, L-tyrosine, L-cystine>beta alanine, L-lysine, and L-cysteine. Additional experiments seemed to indicate that the differences in utilization of amino acids was related to molecular structure other than isomerism.

Lilly and Barnett (1953) in studying the utilization of sugars by fungi employed a medium containing asparagine as the nitrogen source. They grew 57 fungi on this medium, approximately 10 per cent of which were Basidiomycetes.

Hacskaylo, Lilly, and Barnett (1954) in an extensive report on the growth of fungi in nitrogen sources, determined the rate and amount of growth of 25 species of fungi (14 of which were Basidiomycetes) when they were supplied with nitrate, ammonium, and organic (asparagine) nitrogen. They reported that nitrate nitrogen was used slowly by some of the Basidiomycetes tested--with one exception, Polyporus distortus--and poorly, if at all, by the others. From the standpoint of rapidity and the amount of mycelium produced, asparagine and ammonium sulfate supplemented with fumaric acid were about equal in value and superior to either ammonium sulfate or potassium nitrate. They theorized that the steps in the reduction of nitrate to ammonia were the limiting factors, or that adaptive enzyme formation was required for utilization of nitrate nitrogen by these species. They added that the latent ability of an organism to utilize nitrate nitrogen may be easily overlooked when short times of incubation are employed.

In an earlier paper Leonian and Lilly (1940) stated that various organic acids (especially four-carbon dicarboxylic acids or their salts) render the less favorable sources of nitrogen such as arginine and ammonium nitrate very readily available so that often as much as 1000 per cent increase or more in growth is effected.

Evans and Butts (1949) while studying the inactivation of amino acids by autoclaving determined that none of them was significantly

destroyed when autoclaved alone. However, whenever the single amino acids were autoclaved in a solution containing sucrose, over 45 per cent of the amino acid was destroyed (except for cystine).

Experimental

In line with the procedure used in the carbon-source studies, a similar approach was thought to be the best method of obtaining information concerning nitrogen utilization by C. radicata var. furfuracea.

The concentration of nitrogen in basal medium B (source: Bacto-Peptone) had been proved previously to produce a relatively luxurious growth of many organisms. This figure was, therefore, arbitrarily chosen as the standard. Calculation of the quantities needed was accomplished in the following manner:

In one liter of medium B there are five gm. of Bacto-Peptone. The per cent of nitrogen by weight was calculated to be 16.16. The resultant calculation indicates that there are 808 mg. of nitrogen in one liter of medium B, or 32.32 mg. of nitrogen in 40 ml. of medium B.

A total of 25 different nitrogen sources was investigated. Each nitrogen source was set up (in triplicate) as follows: The amount of the nitrogen source that would yield 323.2 mg. of nitrogen was weighed and dissolved in 100 ml. of distilled water. Ten ml. of each stock solution was placed in a separate 125-ml. Erlenmeyer flask. Twenty ml. of double-strength medium B (minus the Bacto-Peptone it normally contained) was added to each flask. Finally, the volume was brought to 40 ml. by adding 10 ml. of distilled water. In the case of casein

hydrolysate, malt extract, dried skim milk, and yeast extract, this procedure was varied because the nitrogen concentrations of these sources were unknown. The figure of 16.16 was used as the arbitrary percentage of nitrogen in these substances. Consequently, in the case of these substances, 0.20 gm. of each of the substances was placed in a separate 125-ml. Erlenmeyer flask (in triplicate). To this was added 20 ml. of double-strength medium B (minus its usual nitrogen source) and 20 ml. of distilled water.

Procedure of preparation and testing was identical with that used in investigating the utilization of carbon sources. The exceptions to this procedure were: 1) the suspension of 406f produced 29 per cent transmission at 550 mμ, 2) the growth period on the shaker was 23 days, and 3) the drying temperature was 97°C.

Seven of the nitrogen compounds employed in the first nitrogen experiment (nitrogen-utilization) barely supported the growth of 406f. Consequently, their broths were not shipped to the Division of Experimental Chemotherapy, Sloan-Kettering Institute for testing. The data pertinent to this experiment appear in Table 14.

The second nitrogen experiment was designed to study the effects of nitrogen sources, incorporated in addition to the main supply of nitrogen, upon 406f growing in medium B. The same sources of nitrogen were tested as in the preceding experiment. The procedure of preparation and testing was identical with that used in investigating the addition of carbon sources. The exceptions to this procedure were: 1) the suspension of 406f produced 17 per cent transmission at 550 mμ,

Table 14

Nitrogen utilization by Collybia radicata var. furfuracea #406f in relation to the tumor-inhibiting properties of in vitro cultures, shaken for 23 days at room temperature.

Nitrogen source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
Inorganic									
Ammonium salts									
ammonium chloride	100 100 090	0.10	4.9	3.6					
ammonium nitrate	090 090 090	0.09	4.8	3.9					
ammonium phosphate	080 080 100	0.09	4.5	3.9					
ammonium tartrate	140 120 140	0.13	5.3	5.3	5.0	0.6	-	1.31/1.16	0/-0.5
Nitrates**									
calcium nitrate	110 100 110	0.11	4.4	4.4	3.0	0.5	-	0.96/0.99	-0.5/-1.0

*The information in this as well as in the following columns was obtained, in tests of filtrates of the culture media, at the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research.

**Except ammonium nitrate, which is listed under ammonium salts.

(continued)

Table 14 - Continued

Nitrogen source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
potassium nitrate	120 110 110	0.11	4.8	4.4	3.0	0.3	-	1.15/0.99	-0.5/-1.0
sodium nitrate	100 100 Contaminated	0.10	5.0	4.3					
Organic									
Amino acids									
L-alanine	140 120 130	0.13	5.0	4.9	>5.0	0.8	-	1.16/1.07	+0.5/+0.5
L-arginine	110 110 110	0.11	4.9	3.6	5.0	0.6	-	1.22/1.33	+2.0/+1.5
L-asparagine	200 200 250	0.22	4.8	3.7	>5.0	0.8	-	0.84/1.03	+1.0/+1.0
L-glutamic acid	100 090 100	0.10	3.4	2.6	5.0	0.6	-	1.21/1.33	+1.0/+1.5
L-histidine	090 090 080	0.09	4.8	4.0	>5.0	0.8	-	1.32/1.33	0/+1.5

(continued)

Table I - Continued

Nitrogen source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
L-leucine	140 150 150	0.15	4.9	4.4	5.0	0.6	-	1.07/1.07	+1.0/+0.5
L-methionine	200 140 140	0.16	4.9	3.9	>5.0	0.8	-	1.24/1.33	+1.5/+1.5
L-proline	130 130 140	0.13	4.9	4.5	>5.0	0.8	-	1.22/1.07	-0.5/-0.5
L-tryptophan	110 110 120	0.11	4.9	4.5	5.0	0.6	-	1.20/0.99	0/-1.0
DL-glycine	150 150 140	0.15	5.1	4.6	>5.0	0.8	-	1.30/1.07	0/+0.5
DL-sarcosine	090 080 070	0.08	1.5	1.5					
DL-serine	260 Contaminated Contaminated	0.26	5.0	4.5	5.0	0.6	-	0.88/0.91	0/+0.5
DL-valine	120 120 Contaminated	0.12	4.5	4.5	>5.0	0.6	-	1.13/0.99	-1.5/-1.0

(continued)

Table II - Continued

Nitrogen source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
Miscellaneous									
casein hydrolysate	100 080 090	0.09	4.4	3.6					
malt extract	560 490 Contaminated	0.53	4.8	4.4	5.0	0.6	-	0.83/0.91	-1.0/+0.5
skim milk (dried)	380 350 410	0.38	5.4	4.5	>5.0	0.6	-	0.71/0.91	+1.5/+0.5
urea	100 100 110	0.10	4.9	7.1					
yeast extract	810 980 Contaminated	0.90	5.6	4.8	5.0	0.6	-	0.80/0.91	+0.5/+0.5
medium B	180 270 260	0.24	5.3	5.2					
medium A	720 680 640	0.68	5.9	5.5					

2) the growth period on the shaker was 20 days, and 3) the drying temperature was 96°C. The data pertinent to this experiment appear in Table 15.

The slight retardation of the test tumor by the broths containing dried skim milk as a nitrogen source (Tables 14 and 15) led to further experiments. A suspension of Collybia radicata var. furfuracea was used to inoculate two 500-ml. Florence flasks containing 125 ml. of medium A. The cultures were grown on the shaker for 21 days at room temperature. At this time, the remaining culture broths were decanted, and replaced with sterile 125-ml. quantities of three-per-cent dried skim milk solution. These flasks were again placed on the shaker. One flask was removed after 12 days, and the remaining flask after 26 days. An extract was prepared from each flask by blending the mycelium in a Waring Blendor with the dried skim milk solution in which it grew. This extract was then filtered, bottled, and merthiolate added. No weights of the mycelium, nor changes in color, appearance, or pH were recorded; the biosynthesis of the tumor-retarding principle was the only criterion of interest. These preparations were negative according to the evaluation of the Sloan-Kettering Institute for Cancer Research.

A final experiment which incorporated the salient findings of the carbon and nitrogen experiments was performed. A suspension of 406f was used to inoculate four 500-ml. Florence flasks containing 125 ml. of medium B minus the usual sugars and peptone. In lieu of the peptone, this medium contained one-half per cent dried skim milk. In addition, one flask contained one-per-cent each of glucose, mannose, and

Table 15

Effects of addition of nitrogen sources to medium B upon Collybia radicata var. furfuracea #406f in relation to the tumor-inhibiting properties of in vitro cultures, shaken for 20 days at room temperature.

Nitrogen source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
Inorganic									
Ammonium salts									
ammonium chloride	270 280 260	0.27	5.5	5.1	>5.0	0.8	-	0.84/0.88	+1.0/+1.5
ammonium nitrate	220 220 200	0.21	5.5	5.1	>5.0	0.8	-	1.50/1.41	-1.5/+1.0
ammonium phosphate	240 240 220	0.23	5.5	5.1	5.0	0.8	-	0.78/0.88	+2.0/+1.5
ammonium tartrate	300 350 340	0.33	5.5	5.1	5.0	0.6	-	0.85/0.93	-0.5/-0.5
Nitrates**									
calcium nitrate	290 260 220	0.26	5.5	5.1	5.0	0.6	-	1.05/0.88	+2.0/+1.5

*The information in this as well as in the following columns was obtained, in tests of filtrates of the culture media, at the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research.

**Except ammonium nitrate, which is listed under ammonium salts.

(continued)

Table 15 - Continued

Nitrogen source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
potassium nitrate	200 230 210	0.21	5.5	5.1	5.0	0.6	-	0.83/0.88	+0.5/+1.5
sodium nitrate	190 210 200	0.20	5.5	5.1	>5.0	0.6	-	0.80/0.88	+1.0/+1.5
Organic									
Amino acids									
L-alanine	290 300 370	0.32	5.5	5.3	>5.0	0.8	-	1.07/0.93	-0.5/-0.5
L-arginine	300 300 320	0.31	5.5	5.3	>5.0	0.6	-	1.05/0.93	+0.5/-0.5
L-asparagine	340 270 320	0.31	5.5	5.2	>5.0	0.8	-	1.03/0.93	-1.0/-0.5
L-glutamic acid	310 260 340	0.30	3.5	3.3	>5.0	0.8	-	1.24/1.16	0/-0.5
L-histidine	300 340 320	0.32	5.2	5.0	>5.0	0.6	-	0.97/0.93	+0.5/-0.5

(continued)

Table 15 - Continued

Nitrogen source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
L-leucine	360 320 340	0.34	5.2	5.0	>5.0	0.6	-	0.95/0.93	-0.5/-0.5
L-methionine	380 370	0.38	5.5	4.9	>5.0	0.8	-	0.87/0.93	-0.5/-0.5
L-proline	280 320 330	0.31	5.5	5.2	>5.0	0.8	-	1.29/1.41	+1.5/+1.0
L-tryptophan	300 270 310	0.29	5.5	5.1	>5.0	0.8	-	1.49/1.41	-0.5/+1.0
DL-glycine	320 300 320	0.31	5.5	5.2	>5.0	0.8	-	1.31/1.41	+0.5/+1.0
DL-sarcosine	120 130 110	0.12	2.0	2.1	3.0	0.5	-	1.42/1.41	+1.0/+1.0
DL-serine	350 340 320	0.34	5.5	5.0	>5.0	0.6	-	0.95/0.93	-1.0/-0.5
DL-valine	260 270 240	0.26	5.5	5.1	>5.0	0.8	-	1.27/1.41	-1.5/+1.0

(continued)

Table 15 - Continued

Nitrogen source	Weight of dried mycelium in mg.	Weight averaged	pH before growth	pH after growth	Mouse toxicity	Dose: cc 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control
Miscellaneous									
casein hydrolysate	280 270 280	0.28	5.2	5.1	>5.0	0.8	-	0.84/0.88	-0.5/+1.5
malt extract	430 480 Contaminated	0.46	5.2	5.1	>5.0	0.6	-	0.86/0.87	0/-0.5
skim milk (dried)	470 620 Contaminated	0.55	5.5	5.1	>5.0	0.6	-	0.71/0.87	0/-0.5
urea	430 490 380	0.43	5.5	6.1	5.0	0.6	-	0.96/0.93	0/-0.5
yeast extract	570 630 Contaminated	0.60	5.5	5.3	>5.0	0.6	-	0.80/0.87	0/-0.5
medium B	300 320 330	0.32	5.5	5.1	>5.0	0.8	-	0.80/0.88	+0.5/+1.5
medium A	490 490 540	0.51	5.5	5.1	>5.0	0.8	-	0.81/0.88	+1.0/+1.5

galactose; one flask contained three per cent mannose; one flask contained three per cent glucose; and the final flask contained three per cent galactose. A similar set of four flasks was set up; these contained one-half per cent yeast extract in addition to the aforementioned compounds.

All eight flasks were placed on the shaker for 31 days. At this time, extracts were made in the manner previously described. No weights of mycelia produced were recorded, since the production of the tumor-retarding principle was the main objective. According to the Sloan-Kettering Institute's evaluation reports, it was not present in any of these eight samples. However, statistical analysis of these results disclosed significant depression of the test tumor in the sample prepared from the flask containing one-half per cent yeast extract plus one-per-cent each of glucose, mannose, and galactose.

The F test from an analysis of variance performed on observations made on the individual mouse tumors also disclosed significant depression of mouse sarcoma 180 by filtrates of the media when dried skim milk served either as the sole nitrogen source (Table 14) or when added to the basal medium (Table 15). The preparations showing significant tumor depression are listed in Table 16.

Table 16

Significant depression of mouse sarcoma 180 after treatment with filtrates of *Collybia radicata var. furfuracea* #406f.

Nitrogen sources in medium	Tumor size expressed as per cent of control tumor
Dried skim milk, 0.5%	78*
Dried skim milk, 0.5%, plus 1% each of mannose, glucose, and galactose, and 0.5% yeast extract	81*
Added to Medium B (see Appendix)	
Dried skim milk, 0.25%	81*

*0.05 level of P

CHAPTER IX

MISCELLANEOUS EXPERIMENTS WITH COLLYBIA RADICATA VAR. FURFURACEA #406f

Several additional lines of interest were investigated. It seemed advisable to test a variety of heterogeneous materials for no particular cogent reason other than to complete the rather exhaustive investigation of various nutrients. Guirard et al. (1946a, 1946b) reported that the presence of sodium acetate stimulated the growth of some microorganisms. Consequently, sodium acetate was added to medium A in four levels (0.5, 1.0, 2.0, and 4.0 per cent) before and after autoclaving. Since Roland and Weiner (1955) reported that Basidiomycetes can readily perform oxygenation and other biomodifications of steroids, cholesterol, lecithin, and oxgall were likewise added to medium A at various levels. Dried egg peptone was substituted at several levels for Bacto-peptone. Two of these preparations showed tumor-retarding activity but further investigations along these lines yielded negative results.

Also investigated at this time was the possible stimulatory effect which vitamins, other than those previously tested, might have upon the elaboration of the active material. Vitamins A, E, and D in various forms were added to medium A. Although a few preparations showed activity when evaluated by the Sloan-Kettering Institute, they were all adjudged ineffective when they could not consistently be reproduced.

Bottomley (1914) reported that an extract of sphagnum peat which had undergone partial decomposition by Azotobacter species stimulated the growth of microorganisms in a manner unmatched by ordinary peat extracts. With this in mind, "bacterized peat" extract, as well as an extract of an Azotobacter sp. were added to medium A. Neither affected the growth or the production of the tumor-retarding material.

Although the initial and final pH levels had been observed and recorded in the carbon and nitrogen experiments, it was of interest to determine how a change of pH during the active growth phase might affect the organism. Accordingly, after 19 days of growth in medium A, the beer from the flasks was decanted and replaced with solutions whose pH ranged from 3.0 to 11.0. At the end of an additional 14 and 28 days on the shaker, the pH of all these flasks was in the vicinity of pH 5.5-8.0. All the flasks showed a slight increase in the amount of mycelium present and none showed evidence of a tumor-retarding principle.

Large volume experiments were also conducted. Organism #406f was grown in one liter of medium A in Fernbach flasks on the shaker and also stationary. It was also grown in three liters of medium A in five-gallon Pyrex carboys on the shaker and in stationary culture. None of the samples prepared from these cultures were active.

Another set of experiments where 406f was grown in Fernbach flasks and Pyrex carboys employed 1) cheesecloth strips and milk filter discs hanging from the necks of these vessels and 2) stainless steel screening rolled into a sphere inside the containers. The organism grew well in all cases. Some of the samples prepared demonstrated tumor-retarding

properties but when replicate experiments were run, the results were not duplicated.

A final experiment, employing kinetin riboside, N^6-benzyl-N^6-methyladenine, and the culture beer of Gibberella fujikuroi, determined that none of these growth stimulators affected either the growth of 406f or its ability to produce the tumor-retarding material.

CHAPTER X

SCREENING OF BASIDIOMYCETE SPOROPHORES FOR TUMOR-RETARDING PROPERTIES

Since Collybia radicata var. furfuracea #406f proved to be no more consistent than organism #288j in producing the tumor-retarding principle, the screening of additional Basidiomycete sporophores was continued. Extracts were prepared by either steeping slices of the sporophore in distilled water, or by blending the sporophore with distilled water in a Waring Blendor for two minutes. Table 17 shows the result of this investigation. The ratio of sporophore to water is given in each case, as is also the method employed in making the extracts. Previous to bottling, the extracts were filtered through Sargent No. 500 filter paper, and merthiolate was added to give a final concentration of one part per 10,000.

The sporophores were identified; in instances where identification of the species was impossible the generic identification is reported. When working with fresh specimens that were in good condition, tissue cultures were attempted, using the context portion of the pileus. Though many of these were contaminated, many pure cultures were obtained.

It should be noted that the screening program covered several seasons. The culture of C. radicata var. furfuracea was chosen for investigation following the experiments on organism #288j because of all the sporophores whose extracts contained tumor-retarding principles, it grew fastest. Boletus Frostii #393 apparently contained a stronger

Table 17

Results of mouse sarcoma 180 tests with extracts of Basidiomycetes.

Species	Procedure of preparation	Origin of material	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control	Deaths
Agaricus placomyces	Fresh sporophore, blended 78:100	Lansing, Michigan	3.0	0.5-0.3	-	0.92/1.10	-1.5/-1.0	
Agaricus rodmani	Fresh sporophore, blended 35:100	Lansing, Michigan	5.0	0.5	-	0.94/1.10	-1.5/-1.0	
Boletinus cavipes	Fresh sporophore, blended 1:2	Lansing, Michigan	0.5	0.5(dil 1-10)	- ±	0.89/0.87 0.71/1.02	-1.0/+1.0 -2.0/0	
Boletus edulis clavipes	Fresh sporophore, steeped 1:2	Lansing, Michigan	RETEST:	0.5 0.5-0.1(dil 1-10)	? -	? 1.07/1.16	? 0/-0.5	4
Boletus edulis pinicola	Fresh sporophore, blended 1:2	Bavaria, Germany	RETEST:	0.5 0.5(dil 1-10)	? ±	? 0.61/0.98	? -2.0/-1.5	5
Boletus fraternus rubellus	Fresh sporophore, steeped 1:2	Lansing, Michigan	5.0	0.5	-	0.85/1.10	-2.5/-1.0	
Boletus Frostii	Fresh sporophore, blended 1:5	Lansing, Michigan	RETEST: RETEST:	0.5 0.5(dil 1-5) 0.5(dil 1-5)	? ± ±	? 0.49/1.09 0.42/0.98	? -2.0/ -3.0/-1.5	5 1 1
Boletus sp. #394	Fresh sporophore, blended 1:3	Lansing, Michigan		0.5	-	0.82/0.90	-2.5/-1.0	
Boletus sp. #629	Fresh sporophore, blended 1:2	Albany, Wyoming	3.0	0.3	±	0.74/0.99	-1.5/-2.0	
Boletus sp. #630	Fresh sporophore, blended 1:2	Albany, Wyoming	1.0 RETEST: RETEST:	0.5(dil 1-2.5) 0.5 0.5	- ± -	0.84/0.99 0.73/1.02 0.82/1.07	-0.5/-2.0 -3.5/-0.5 -2.0/-1.0	2 2 2

*The information in this as well as in the following columns was obtained from the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research.

(continued)

Table 17 - Continued

Species	Procedure of preparation	Origin of material	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control	Deaths
Calvatia maxima	Fresh sporophore, blended 1:2	Lansing, Michigan	0.5(dil 1-10)	0.6(dil 1-100)	±⁻	0.49/0.87	-2.0/+1.0	
			RETEST:	0.5(dil 1-100)	±⁻	0.68/1.10	-1.5/+0.5	
Calvatia maxima #642	Fresh sporophore, blended 1:2	Lansing, Michigan	0.5	0.6(dil 1-20)	±⁺	0.23/0.68	-3.5/+2.5	1
			RETEST:	0.5(dil 1-20)	?			3
Calvatia maxima #643	Fresh sporophore, blended 1:2	Lansing, Michigan	0.5	0.5(dil 1-25)	?			4
			RETEST:	0.5(dil 1-50)	Not graded			
			RETEST:	0.5(dil 1-100)	±⁻	0.73/1.20	-1.5/-3.5	1
Calvatia maxima #644	Fresh sporophore, blended 1:2	Lansing, Michigan	0.5	0.5(dil 1-20)	±⁺	0.25/0.68	-3.5/+2.5	2
			RETEST:	0.5(dil 1-2)	?			4
			RETEST:	0.5(dil 1-20)	?			5
Calvatia maxima #645	Fresh sporophore, blended 1:2	Lansing, Michigan	0.5	0.5(dil 1-20)	±⁺	0.30/0.68	-1.5/+2.5	1
			RETEST:	0.5(dil 1-10)	?			3
			RETEST:	0.5(dil 1-20)	±⁻	0.72/1.10	-4.5/-1.0	1
Calvatia maxima #646	Fresh sporophore, blended 1:2	Lansing, Michigan	0.5	0.5(dil 1-10)	?			4
			RETEST:	0.5(dil 1-20)	Not graded			
			RETEST:	0.5(dil 1-60)	±⁻	0.89/1.20	-2.5/-3.5	
Calvatia maxima #647	Fresh sporophore, blended 1:2	Lansing, Michigan	0.5	0.5(dil 1-25)	±⁻	0.21/0.68	-2.5/+2.5	1
			RETEST:	0.5(dil 1-20)	?			4
Collybia radicata var. furfuracea	Fresh sporophore, blended 1:1	Lansing, Michigan		0.5	±⁻	0.64/0.90	-2.0/-1.0	
			RETEST:	0.5	-	0.82/0.98	-3.0/-1.5	
Clitopilus abortivus (non-abortive form)	Fresh sporophore, blended 1:2	Lansing, Michigan	3.0(dil 1-10)	0.5(dil 1-10)	-	0.96/0.91	-1.5/0	
			RETEST:	0.5-0.4(dil 1-5)	-	0.95/1.02	-2.5/0	
Clitopilus abortivus (abortive form)	Fresh sporophore, blended 1:1	Lansing, Michigan	1.0	0.5(dil 1-5)	-	0.91/0.91	0/0	
			RETEST:	0.5(dil 1-2.5)	-	0.86/1.02	-3.0/0	1
Collybia velutipes	Fresh sporophore, blended 1:6	Lansing, Michigan		0.5-0.2	?	?	?	3
			RETEST:	0.5-0.25(dil 1-5)	-	1.01/1.07	+0.5/-1.0	

(continued)

Table 17 - Continued

Species	Procedure of preparation	Origin of material	Mouse toxicity*	Dose: cc. 2x daily	Effect	Tumor diam. in cm./diam. of control tumor	Wt. change in gm./wt. change of control	Deaths
Cortinarius alboviolaceus	Fresh sporophore, blended 1:2	Lansing, Michigan	1.0	0.5-0.4(dil 1-10)	-	0.91/0.91	-1.5/0	1
Hydnum septentrionale	Fresh sporophore, blended 1:2	Lansing, Michigan		0.5	?	?	?	5
			RETEST:	0.5	±	0.76/1.09	-1.5/unknown	
			RETEST:	0.5(dil 1.10)	-	0.81/0.98	0.5/-1.5	
Hydnum sp. #445	Fresh sporophore, blended 1:10	Lansing, Michigan	3.0	0.5	-	0.80/0.92	-2.0/-2.5	
Hydnum sp. #446	Fresh sporophore, blended 1:6	Lansing, Michigan	5.0	0.5	±	0.66/0.92	-3.5/-2.5	
Hydnum sp. #654	Fresh sporophore, blended 1:3	Lansing, Michigan	5.0	0.6	?			3
Hypholoma sublatericum	Fresh sporophore, blended 1:1	Lansing, Michigan		0.5-0.2	?	?	?	2
			RETEST:	0.2-0.1	-	0.95/1.07	+0.5/-1.0	
Leptiota americana	Fresh sporophore, blended 1:2	Lansing, Michigan	0.5	0.5(dil 1-10)	-	1.01/0.87	+0.5/+1.0	
			RETEST:	0.5(dil 1-5)	-	0.92/1.02	+2.0/0	
Lepiota rhacodes	Fresh sporophore, blended 1:2	Lansing, Michigan	0.3	0.3	-	0.76/0.91	+0.5/0	
Polyporus (Xanthochrous) nigricans	Fresh sporophore, blended 1:3	Lansing, Michigan	1.0	0.5-0.4(dil 1-2.5)	-	1.19/1.05	-0.5/+1.0	
			RETEST:	0.5	±	0.69/0.93	+2.0/+1.5	
Psathyra sp. #472	Fresh sporophore, blended 1:1	Lansing, Michigan	3.0	0.6(dil 1-10)	-	1.14/1.14	+1.5/+1.0	
Boletus edulis var. pinicola	Dried sporophore, steeped 1:10	Bavaria, Germany	0.5	0.5-0.2(dil 1-2.5)	±	0.46/0.90	-5.5/-1.5	1
Boletus edulis	Dried sporophore, steeped 1:10	Chile	3.0	0.3	-	1.16/1.05	-1.5/-1.5	
Cortinellus shiitake	Dried sporophore, steeped 1:10	China	3.0	0.5	?			4
			RETEST:	0.5(dil 1-3)	±	0.74/1.09	-0.5/0	1
Cortinellus shiitake	Dried sporophore, steeped 1:10	China		0.5-0.25	?			4
			RETEST:	0.5(dil 1-3)	±	0.62/1.02	-2.0/-1.0	1

tumor-retarding material, but the culture was extremely slow-growing, and eventually ceased to grow. Cultures of Boletinus cavipes, Boletus edulis pinicola, Hydnum septentrionale, Hydnum sp. #446, and Polyporus nigricans could not be obtained owing to the presence of parasitic microorganisms, or else the dearth of viable cells.

Thirteen of the 31 extracts prepared from the fresh sporophores and three of the four extracts prepared from dried sporophores showed the presence of tumor-retarding principles in the mouse sarcoma 180 tests made by the Sloan-Kettering Institute for Cancer Research.

CHAPTER XI

EXPERIMENTS WITH *CALVATIA MAXIMA* #642

Water extracts of fresh sporophores of *Calvatia maxima* strains #642-647 had shown the presence of an oncostatic agent in mouse sarcoma 180 tests at the Sloan-Kettering Institute. Cultures of all six of these isolations were viable, and strain #642 was chosen for *in vitro* work. The primary interest was the same as in the *Collybia* experiments, namely to determine whether the tumor inhibitor encountered in sporophores would also be produced consistently *in vitro*. This was found to be the case when preparations made from cultures of #642 were grown in medium A. The main problem now appeared to be not one of nutrition but one of determining the optimum environmental conditions under which the tumor-retarding material would be produced consistently.

A preliminary study established that *C. maxima* #642 grew at temperatures ranging from 8° to 28.5°C., with the optimum temperature range between 20° and 26°C. This was determined by placing 3.5 mm. plugs on triplicate medium A agar plates at temperatures of 8, 12, 16, 20, 22, 24, 26, 28, 30, and 37 degrees C. and recording the diameter of each colony. The averaged resultant values are shown diagrammatically (Figure IV).

The elaboration of the tumor-retarding material was investigated by using shake cultures of #642 at five temperature levels, 16, 19, 22,

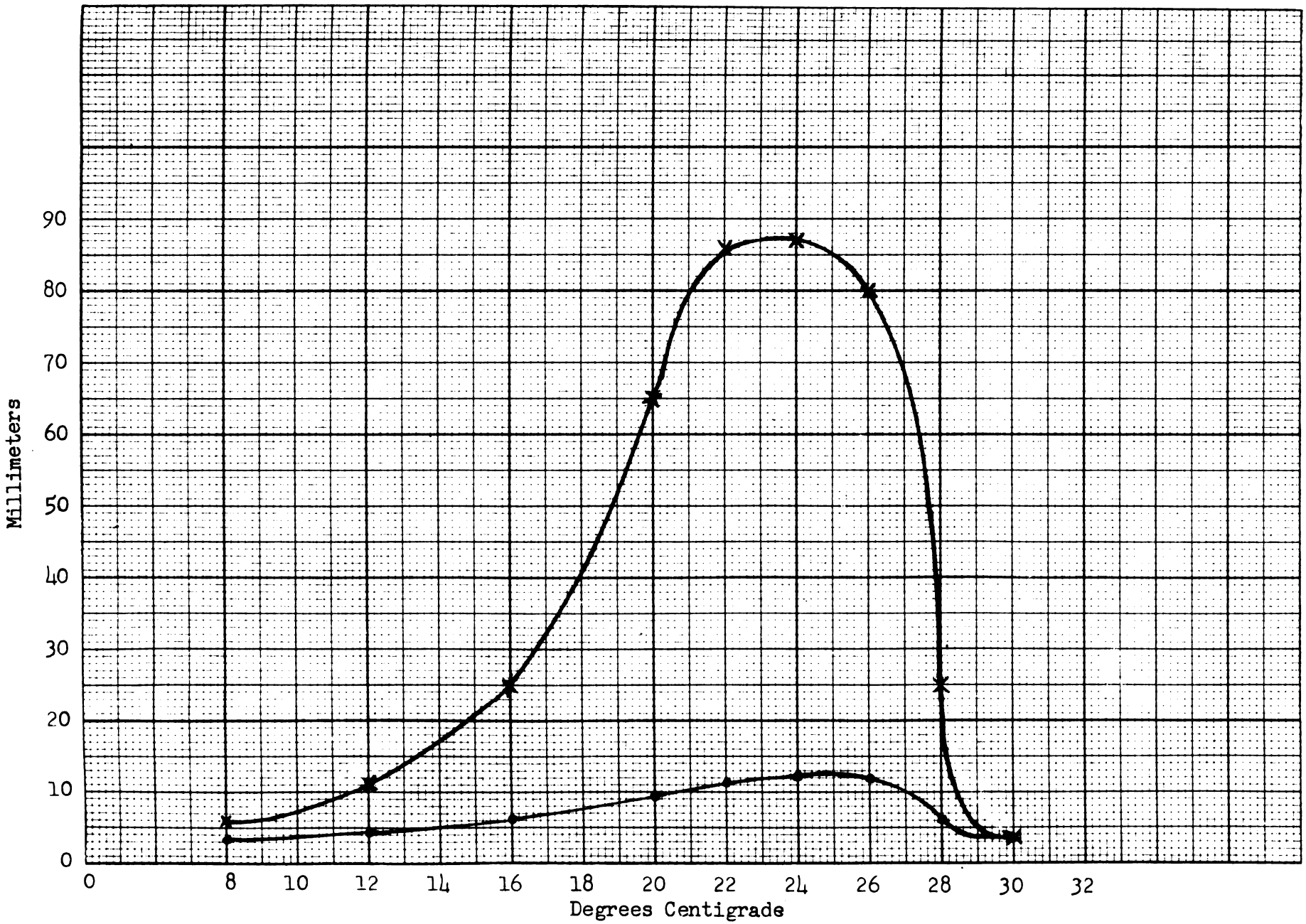

Figure IV. Diameter of mycelial colonies of Calvatia maxima #642 grown on medium A agar plates.

7-day growth 28-day growth

25, and 28 degrees C. The 19°C. experiment is described and serves as an example of the temperature study.

Thirty-five 500-ml. Florence flasks containing 125 ml. of medium A broth were inoculated with 10 ml. of a suspension of C. maxima #642 according to the procedure previously described, and placed on the shaker in a controlled temperature room at 19°C. Starting with the fourth day, and subsequently at four-day intervals (until the 36th day), three flasks were removed from the shaker. Samples were prepared in the manner previously described by blending the mycelium with the culture beer and were shipped to the Sloan-Kettering Institute for evaluation. The results of the individual series were averaged (Table 18) and plotted (Figure V). The eight excess flasks were inoculated as alternates in case any flasks should become contaminated.

Table 18

Results of mouse sarcoma 180 tests with *in vitro* cultures of *Calvatia maxima* #642 grown at 19°C.

Sample Number	Days Old	Effect*	Tumor diam. in cm./ diam.of control tumor	Per cent Change	Per cent Average
1	4	-	1.00/0.89	+12.3	
2	4	-	0.89/0.87	+2.2	+7.1
3	4	-	0.93/0.87	+6.8	
4	8	-	1.04/1.28	-18.8	
5	8	-	1.20/1.28	-6.3	-18.0
6	8	±	0.91/1.28	-29.0	
7	12	±	0.73/1.15	-36.6	
8	12	-	1.04/1.15	-9.6	-19.2
9	12	-	1.02/1.15	-11.3	
10	16	±	0.68/0.91	-25.3	
11	16	±	0.68/0.91	-25.3	-25.7
12	16	±	0.67/0.91	-26.4	
13	20	±	0.71/1.16	-38.8	
14	20	±	0.75/1.10	-31.9	-29.3
15	20	-	0.91/1.10	-17.3	
16	24	±	0.75/1.35	-44.5	
17	24	±	0.90/1.35	-32.4	-39.5
18	24	±	0.79/1.35	-41.5	
19	28	±	0.76/1.05	-27.7	
20	28	±	0.55/1.05	-47.7	-36.6
21	28	±	0.69/1.05	-34.3	
22	32	-	1.06/1.38	-23.2	
23	32	±	1.01/1.38	-26.9	-27.6
24	32	±	0.93/1.38	-32.7	
25	36	-	0.94/0.94	0	
26	36	-	0.76/0.94	-19.2	-9.6
27	36	-	0.85/0.94	-9.6	

*The information in these columns was obtained from the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research.

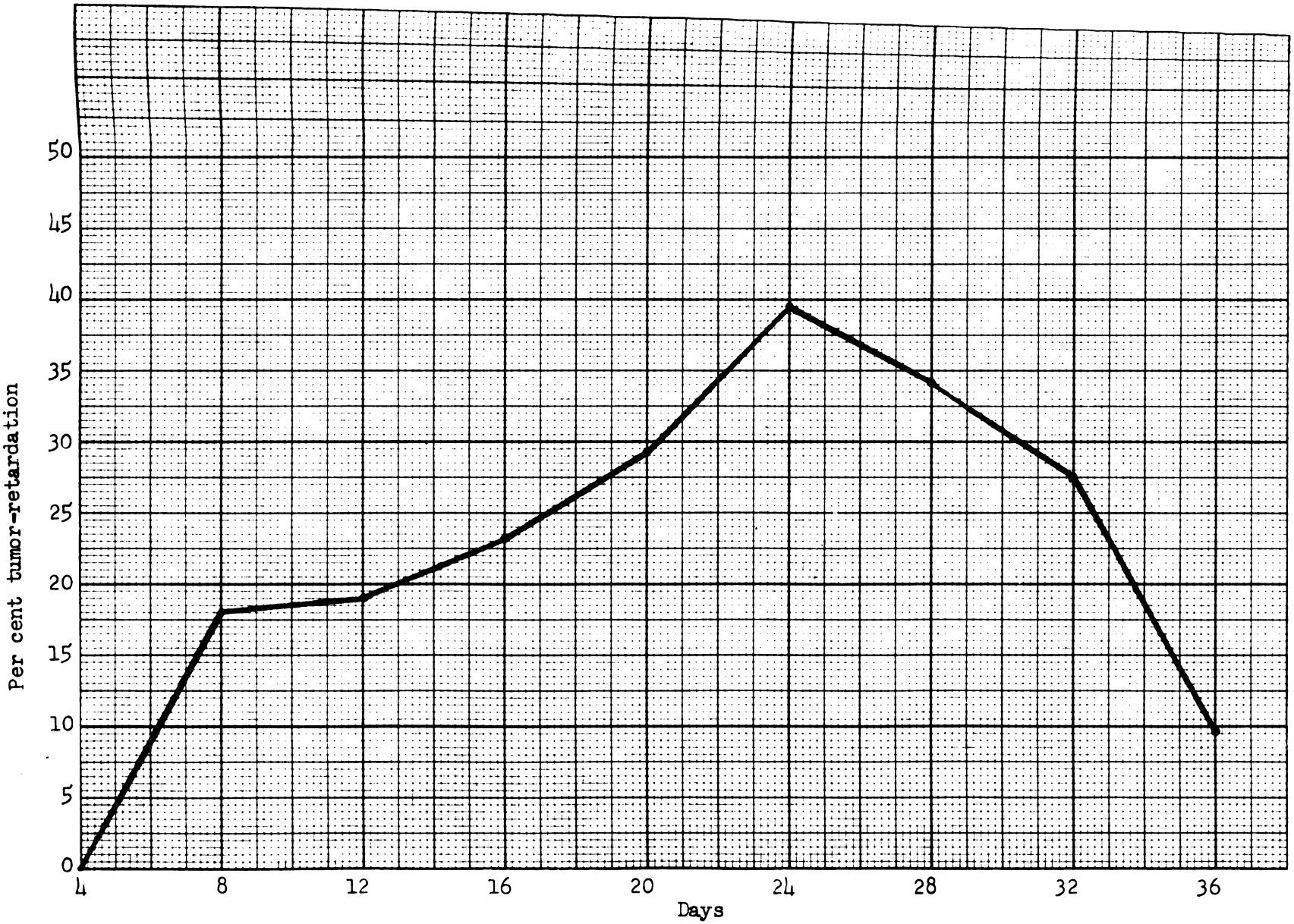

Figure V. Elaboration of a tumor-retarding principle in shake cultures of Calvatia maxima #642 grown at 19°C. Determined at the Division of Experimental Chemotherapy of the Sloan-Kettering Institute for Cancer Research with mouse sarcoma 180.

CHAPTER XII

DISCUSSION OF RESULTS

Experiments with Basidiomycete #288j

Evidence had been obtained through the experiments conducted upon Basidiomycete #288j that the tumor-retarding principle can be elaborated by the mycelium *in vitro*. The control of environment, however, which is indispensable for this purpose, has not been accomplished as yet and will require further investigation.

Preliminary Experiments with *Collybia radicata* var. *furfuracea* #406f

While trying to discover an explanation for the inconsistent elaboration of the tumor-retarding principle, it was noted that the flasks had been inoculated with a culture of 406f which was the fourth consecutive transfer on artificial media the organism had undergone. Derrick (1949) mentioned that often Basidiomycete cultures slow down their growth rate or die after the second through the ninth consecutive transfer on laboratory media. This may be part of the answer to the organism's apparent loss of the ability to produce the tumor-retarding principle *in vitro*. However, it should be noted that in the case of this organism there was no apparent decrease in the rate of growth, merely a decrease in the measurable quantity of the production of the tumor-retarding material, i.e., a physiological change had occurred.

The Effects of Nutrilites upon *Collybia radicata* var. *furfuracea*

Of the three experiments reported using nutrilites, the last (Table 5) is perhaps the most important. Lindeberg's solution B, being a purely synthetic medium, was formulated with the idea in mind that any response by the test organism in the presence of a nutrilite could be easily demonstrated.

The results of this experiment indicate that 406f will grow without nutrilites present. None of the 18 nutrilites tested elicited a response better than that seen in the control flasks containing Lindeberg's solution B. Certainly there was nothing like the 15- to 25-fold increase Lindeberg reported for *Collybia ambusta*, *C. butyracea*, or *C. velutipes* when thiamine was added. The conclusion that could be drawn, therefore, is that 406f neither requires nor is stimulated by the nutrilites tested. This, of course, brings up the question of why 406f grows better when Bacto-Peptone or Bacto-Yeast Extract is present in the medium. Although there is no positive proof, we can suggest a possible answer. Marczynski (1943) thought that this might be due to the presence of more assimilable nitrogen. Fries (1948) said, "The (yeast) extract in question may serve as a buffer to the solution, it may contain metals, which are present in the solution in insufficient amounts, and it may contain carbon-, nitrogen-, or sulphur-compounds which are more easily assimilated. There is, however, no doubt that the effect in many cases is brought about by growth factors of a sort as yet unknown."

These are but a few explanations regarding the stimulatory action of Bacto-Peptone and Yeast extract. Fries' idea concerning the unknown growth factors is entirely plausible when we consider the definition of a growth factor, namely "a substance which is essential for optimum growth and is effective in minute quantities."

Other investigators have also reported on the effects of unnamed growth factors. Robbins (1939) reported the presence of unknown growth factors in agar, brown sugar, potatoes, corn meal, and oatmeal. These unknown substances stimulated 1) the growth and 2) spore germination of Phycomyces Blakesleeanus. In a later paper (1941) he reported the presence of the same factors (now labelled Z_1 and Z_2) in Neo-Peptone and Difco Agar. Z_1 was found not to be identical with biotin, pantothenic acid, glutamine, or para-aminobenzoic acid. Z_2 was found not to be identical with glutamine or para-aminobenzoic acid.

Melin (1946) reported that forest litter extracts increased the in vitro growth of Lactarius deliciosus up to 60 times. He suggested that the active principle in litter extract might be identical with Robbins' factor Z, and theorized that it might be hypoxanthine. He tested species of the genera Boletus, Lactarius, Paxillus, Rhizopogon, Morchella, Psalliota, Clavaria, Clitocybe, Collybia, Mycena, and Stropharia (all mycorrhiza-forming fungi), and found that most species required thiamine, and a few needed biotin in addition. Litter extracts increased growth 150-300 per cent in some cases. He also reported that some leaf extracts (maple, birch, beech, oak, aspen, and pine) inhibited growth. Some were intensified in their inhibitory action after being autoclaved.

Hawker (1936) reported that while Collybia velutipes grew and fruited in light on a mineral-dextrose medium containing potassium nitrate and 1.5 per cent agar, the addition of a lentil extract increased fruiting.

None of these investigators associated their unidentified growth factors with either thiamine or riboflavine.

On the other hand, Robbins and Schmidt (1938) have proved that Neo-Peptone, a product of the Digestive Ferments Company contains a small amount of thiamine or its intermediates (about 0.00002 per cent, enough to cause growth of excised tomato roots). Thus, if five grams of Neo-Peptone were added to one liter of medium, 100 micrograms of thiamine would be present in that quantity of medium.

An analysis furnished us by the Difco Company (Detroit, Michigan) on their product Bacto-Peptone (see Appendix) shows it to contain 2.50 micrograms of pyridoxine, 0.32 micrograms of biotin, 0.50 micrograms of thiamine, 35.00 micrograms of nicotinic acid, and 4.00 micrograms of riboflavine per gram.

Since five grams of this product comprise part of the formula of basal medium B, it can be seen that this medium contains 2.50 micrograms of thiamine per liter. This is far below the amount of thiamine reported by other investigators as being necessary for the growth of certain basidiomycetous mycelia.

An analysis furnished by the Difco Company on their product Bacto-Yeast Extract (see appendix) shows it to contain 20.0 micrograms of pyridoxine, 1.4 micrograms of biotin, 3.2 micrograms of thiamine,

279.00 micrograms of nicotinic acid, 19.00 micrograms of riboflavine, and 0.3 micrograms of folic acid.

Since five grams of this product comprise part of the formula of our medium A, this medium therefore contains 16 micrograms of thiamine per liter. This is less than one-third of the amount of thiamine that Lindeberg reported was necessary to stimulate the growth of Collybia ambusta, C. butyracea, and C. velutipes 15- to 50-fold.

The conclusion one must draw then is that none of the nutrilites when used singly has any stimulatory action upon culture #406f. However, it would be wise to note that in Bacto-Peptone and Bacto-Yeast Extract there are not single nutrilites present, but rather several in combination with several amino acids, several salts, proteoses, and peptones. When they are present in proper balance they may (and only then) exert an effect which is not seen when they are investigated singly.

Lewis (1953) believes that a balance exists between the substances that promote and those that inhibit the growth of an organism. He also pointed out that it is important to remember that, while one nutrient may be essential or stimulatory to one species of a specific genus, it may actually inhibit a second species of the same genus.

Some of these ideas may help explain the results of the first nutrilite experiment reported (Table 3). Thiamine, riboflavine, pyridoxine, ribose, and nucleic acid all show at least a 20 per cent increase in dry mycelial weight over the control flasks (Basal medium B). The same nutrilites, when added to Lindeberg's solution B, (Table 5)

produced no stimulatory effects. Perhaps it is the new balance which the specific nutrilite affects in the presence of the assorted nutrients (see Appendix for ingredients of basal medium B and analysis of Bacto-Peptone) that produces the stimulation. These same nutrilites (except ribose) repeat this stimulatory effect under the similar conditions reported in the second experiment (Table 4).

Skoog (1954) reported that evidence exists to implicate nucleic acids directly or indirectly as key substances in the chemical regulation of growth. This may explain the action of the nucleic acid preparation used (Table 3).

The Effects of Carbon Sources upon *Collybia radicata* var. *furfuracea*

Considering that all the carbon sources tested were present in an amount calculated to yield an equal supply of carbon, some interesting results were observed when the results were tabulated. Judging from the first experiment (carbon utilization) the trisaccharides apparently were most conducive, as a group of carbon sources, to the production of mycelium by *C. radicata* var. *furfuracea*. The disaccharides were next best, followed closely by the monosaccharides (the hexoses being better than the pentoses). The single polysaccharide tested, dextrin, was next best. The lowest yields were obtained with polyhydric alcohols, $NaHCO_3$, the single inorganic salt used, and the organic acids.

There are many possible explanations; an examination of the components of the poly-, tri-, and disaccharides may be informative.

Poly-(tri-, or di-,)saccharide	Simple Sugars Present
Dextrin	Glucose
Melezitose	Glucose (2), Fructose
Raffinose	Glucose, Fructose, Galactose
Honey	Glucose, Fructose
Invert sugar	Glucose, Fructose
Lactose	Glucose, Galactose
Maltose	Glucose (2)
Sucrose	Glucose, Fructose
Trehalose	Glucose (2)

It is difficult to comprehend why, for example, melezitose produces a greater mycelial yield than either of its hydrolytic products (glucose and fructose) especially when the organism must first produce an enzyme to hydrolyze this trisaccharide. The same holds true for maltose, sucrose, and invert sugar. Perhaps the explanation may lie in the consideration that the biochemical mechanism which enables the organism to break down the molecule furnishes a stimulus which continues to act after the initial hydrolysis, and thus makes the organism capable of utilizing the resulting monosaccharides at a faster rate than if those carbon sources were available originally in that form.

As previously stated, among the monosaccharides the hexoses were better utilized than the pentoses. The best of these was galactose, followed by glucose, mannose, fructose, and sorbose, in this order. The appearance of galactose as the hexose best utilized by C. radicata

var. furfuracea was certainly unexpected and rather difficult to explain in view of the findings reported by other investigators concerning various fungi. It can only serve to illustrate the variability of preferences among fungi.

Among the pentoses, xylose appeared to be the best utilized, followed by l-arabinose, rhamnose, ribose, and d-arabinose, in this order. The result obtained with xylose appears to confirm the findings of Lutz (1925), Styer (1930), and Treschow (1944).

Disregarding groupings, the (ten) carbon sources best utilized by C. radicata var. furfuracea were melezitose, galactose, sucrose, xylose, invert sugar, maltose, glucose, mannose, l-arabinose, and raffinose, in this order.

The small amount of mycelium produced by culture #406f when polyhydric alcohols and organic acids were used as carbon sources is attributed to the respective high and low pH values observed in the culture media.

As shown in Table 6, the quantity of mycelium produced is not necessarily accompanied by the production of the tumor-retarding principle. Only two carbon sources, both hexoses (glucose and mannose), enabled the organism to biosynthesize measurable concentrations of the active principle (as determined by the Sloan-Kettering Institute for Cancer Research).

In order to obtain more information concerning the effect of the media a statistical analysis of the results was made. It became evident that some of the media supported the organism in elaborating the

tumor-retarding principle although according to the Sloan-Kettering rating this did not constitute tumor retardation. Significant depression of the test tumor was noted at the 0.05 level of probability when the filtrates of the culture media containing citric acid or honey (as the carbon sources) were injected into the mice.

The reason why the additional system of evaluation was used to supplement the Sloan-Kettering method was that in our tests the same compounds and concentrations of certain nutrients retarded the test tumors in repeated consecutive experiments. Since it appeared that some of these replications consistently depressed tumor growth (without bringing about a depression to the level where the $\pm$ rating of the Sloan-Kettering Institute could be given), it was felt that a statistical analysis would be helpful.

The results of the second experiment (carbon sources added to the basal medium) indicate that the disaccharides, as a group, were best utilized by culture #406f judging from the amount of mycelium produced. The monosaccharides were next best, closely followed by the polysaccharides tested. The trisaccharides and polyhydric alcohols followed, and the organic acids and sodium bicarbonate were least useful.

Invert sugar, sucrose, and lactose were the best of the disaccharides, in this order.

Among the monosaccharides, the hexoses produced more mycelium than the pentoses and the others. Fructose was the best of the hexoses, followed by galactose and glucose. The yields produced by the pentoses were very nearly similar. However, xylose and d-arabinose were the best in this group.

The only significant growth produced by a member of the other monosaccharides was provided by alpha-d-glucose-pentaacetate. Melezitose, dextrin, and glycerol were the most important of the trisaccharides, polysaccharides, and polyhydric alcohols, respectively.

Among the organic acids used, uric acid produced twice as much mycelium as the next best carbon source.

Disregarding the individual groupings, the added carbon sources best utilized by 406f were invert sugar, sucrose, fructose, lactose, galactose, glucose, dextrin, xylose, d-arabinose, rhamnose, sorbose, honey, glycerol, ribose, maltose, uric acid, l-arabinose, mannose, alpha-d-glucose-pentaacetate, melezitose, and trehalose, in this order.

Only three of the 40 carbon additives tested made culture #406f biosynthesize a tumor-retarding principle. These were galactose, malonic acid, and sodium bicarbonate.

When statistical analysis was applied to the remaining data, it became apparent that the filtrates of the additives, mannose and invert sugar, caused a significant depression of the test tumor at a 0.05 and 0.01 level of probability respectively.

When the findings of the first carbon experiment revealed that mannose and glucose enabled culture #406f to biosynthesize a tumor-retarding substance, confirmation was essential, as reported previously. The activity of these broth replications did not reach the level on which a positive rating, according to the Sloan-Kettering method of evaluation, could be based. However, statistical analysis of the data showed both of these preparations to be significant at the 0.05 level

of probability. In addition, a preparation made by extracting the mycelium of culture #406f (grown in mannose) with water, was given a $\pm^-$ rating (Table 8).

Although none of the additional experiments with glucose yielded a tumor-retarding principle (as recorded by the Sloan-Kettering method or statistical analysis of the data supplied by that institute), $\pm^-$ ratings were obtained for two preparations made from additional experiments with mannose (Table 9). None of the other preparations proved to have present a tumor-retarding material. Even though at times the test tumor appeared depressed, statistical analysis of these data did not show significance.

Finally, it should be pointed out that three hexoses seem to play an important role in the biosynthesis of the tumor-retarding principle: galactose, glucose, and mannose. In contrast to these, fructose--utilized by 406f not quite as well as the others--never gave any indication of being involved in the biosynthesis of a tumor-retarding principle. This may be ascribed to the fact pointed out by Margolin (1942) that the first three are aldehydes, while fructose is a ketone. However, it should be kept in mind that the molecular configurations of glucose, fructose, and mannose are so very similar that they can be regenerated from either form.

The one very apparent fact that resulted from this investigation of carbon sources is that mannose, more consistently than glucose or galactose, though not always, caused C. radicata var. furfuracea to biosynthesize a tumor-retarding principle. Perhaps future experiments,

where the extracts could be prepared in larger volume and then concentrated by low-pressure evaporation, will prove the contention that this organism can produce such a material *in vitro*. Certainly the results that were obtained, and especially the statistical data, seem to indicate the presence of such a principle. A concentration of the extracts (as suggested above) may intensify the weak action of such a material to a degree where the Sloan-Kettering method of evaluation will always record a $\pm^{-}$ rating.

Sugar Determination

Since there was a correlation between the biosynthesis of the tumor-retarding principle and the presence of certain sugars in the culture medium, it appeared to be of interest to know the sugar content of *C. radicata var. furfuracea* mycelium produced *in vitro*. The possibility was considered that a supply of a simple nutrient like glucose would enable the fungus to synthesize the active material at an accelerated rate.

Hydrolysis of trehalose was observed to occur more quickly (within 30 minutes) and thoroughly (yielding 98 per cent) under pressure in an autoclave then when boiled for six hours (yielding 93.5 per cent) as suggested by Iwanoff (1925).

The carbohydrate content of *A. campestris* as determined by the method employed was found to be approximately only one-tenth the figure reported in the Handbook of Chemistry and Physics (Hodgman, 1950); the figure obtained for the *in-vitro* produced mycelium of *C. radicata var.*

furfuracea was similar to that determined for A. campestris. The figure obtained for these two materials apparently was more in line with the evaluation presented by Gussow and Odell (1927) who stated that "small amounts of nitritive salts and sugars" were contained in A. campestris.

The carbohydrate content of the context was determined to be less than half of that found for the whole mushroom. No explanation, other than to compare this variation with similar variations occurring in distinctive tissues of higher plants, is attempted.

The tissue of C. radicata var. furfuracea grown in vitro appeared to have a sugar content comparable to that of the commercially cultivated A. campestris. Not only have the results of previous tests shown that the elaboration of the active principle is not affected by the presence of excess sugars, but it was noted that no excess sugar was accumulated in the tissue.

The moisture content of A. campestris was difficult to obtain accurately because evaporation occurs while the weighing takes place. It is easy to understand the variation occurring in the literature concerning moisture content when one observes this fast drying. The results obtained by different investigators may have differed because of the evaporation that occurred between the time the specimens were collected and the time they were evaluated. Another possibility for the varying figures is the moisture content of the specimens at the time of collection.

The Effects of Nitrogen Sources Upon *Collybia radicata* var. *furfuracea* #406f

Using the weight of the mycelium produced as the criterion, the individual nitrogen sources best utilized (as established in Table 14) by *C. radicata* var. *furfuracea* were yeast extract, malt extract, dried skim milk, DL-serine, and L-asparagine, in this order. As a group, the miscellaneous nitrogen sources were far better utilized than the amino acids or inorganic forms investigated. In this respect the findings reported here correspond with those of LaFuze (1937) and Derrick (1949).

When the nitrogen sources studied were employed as additives similar results were obtained, judging by the dry weights of the mycelia produced. The best single nitrogen sources were yeast extract, dried skim milk, malt extract, urea, L-methionine, DL-serine, L-leucine, and ammonium tartrate, in this order. Again the miscellaneous group of nitrogen sources produced more mycelial growth than did the amino acids or the inorganic sources. However, in this case, the difference was not as great as it had been previously.

None of the "beers" shipped to the Sloan-Kettering Institute for evaluation contained enough of a tumor-retarding principle to receive a rating of $\pm^{-}$. The slight retardation of the test tumor evoked by the "beers" when dried skim milk was used as either the sole nitrogen source or a nitrogen additive was not duplicable. Similarly, the results obtained using extracts of the combined carbon- and nitrogen-sources media were negative. Although two of the eight samples showed

a slight retardation of the tumor, the results were judged to be inconclusive as they were not readily duplicated.

In any case it appears safe to state that the nitrogen sources studied were not used by C. radicata var. furfuracea to produce the tumor-retarding principle either directly or indirectly.

Miscellaneous Experiments with Collybia radicata var. furfuracea

None of the final experiments with C. radicata var. furfuracea demonstrated activity of a nature that was reproducible. However, one item of major interest was observed in the experiments where cheesecloth strips and milk filter discs were suspended from the necks of the flasks. The strips and discs were supported in such a manner that the bottom edge was submerged in medium A. A suspension of culture #406f was used to inoculate the entire length of the strips and discs. What proved of interest was the growth of the mycelia along the upper portions of the strips and discs. This mycelium was not unlike that of the context of C. radicata var. furfuracea sporophores. It was firm and "snapped" apart when bent, quite unlike the cottony mycelia seen growing on the surface of agar plates or broth cultures, and also very different from the flaccid, wet spheres observed in shake cultures. The samples prepared from these mycelia, however, were also negative.

Screening of Basidiomycete Sporophores for Tumor-Retarding Properties

These preliminary tests cannot determine in any manner whether the active compound is the same in all cases. Certainly the intensities of

the tumor-retarding principles vary, as evidenced by a comparison of the results obtained with Boletus Frostii or Calvatia maxima and those obtained with Collybia radicata var. furfuracea or Hydnum septentrionale (Table 17).

Experiments with Calvatia maxima #642

While determining the temperature range and optimum temperature for the growth of C. maxima #642, a very marked effect was noted in the 28-30°C. range. This organism remained viable at 28.5°C., producing a colony approximately one-third of that observed when it was grown in the 22-26°C. range. The thermal death point of the organism apparently was between 29 and 30°C. The growth that occurred in the lower temperature range--although not spectacular--was fairly uniform.

As shown in Figure V and Table 18, the tumor-retarding principle becomes evident in the samples prepared from the eight-day culture and gradually intensifies until a ± rating is observed in the samples prepared from the 16-day cultures. Figure V demonstrates (in the 8- to 16-day area) the validity of the author's contention in employing statistical analysis on some of the carbon and nitrogen experimental data presented earlier. It was stated then that certain preparations gave an indication of possessing tumor-retarding properties, even though the evaluation by the Sloan-Kettering Institute resulted in a negative rating.

At 19°C., C. maxima #642 demonstrated maximum production of the tumor-retarding principle at 24 days. The active material gradually

lessened--although still evident in the samples prepared from the 32-day cultures--until it was no longer evident in the 36-day cultures, possibly suggesting autolysis.

The 19°C. temperature was found to be the optimum condition for the production of mycelium coupled with the elaboration of the tumor-retarding principle. For this reason only the 19°C. study is presented. In the other four temperature experiments, the tumor-retarding principle first appeared in the samples prepared from the 24- or 28-day cultures.

The original purpose of the work undertaken was the *in vitro* production of tumor-inhibitory principles by Basidiomycetes. The principle first encountered in *Boletus edulis* sporophores was thought to be suitable for experimentation. As this study progressed it became obvious that the difficulties, although they were not considered insurmountable, were of such nature that a solution of the problem would require more time than could be allowed for the performance of a thesis research.

The substitution of a different organism which had also shown tumor-retarding properties in sporophore preparations was therefore considered. *Collybia radicata var. furfuracea* was selected. It should be emphasized that this selection was made on the basis of information at hand at that time and that it represented the best possible choice available. As in every experimental study a risk had to be taken which, as it turned out later, became quite critical. The extensive cultural studies made with this organism failed to produce conclusive information as to its exact requirements, and it remains to be seen

whether this problem can be solved at all. The sporadic production of active extracts shows that the tumor-retarding principle can be produced in cultures of the organism. That it has been impossible to produce it consistently may be due to experimental shortcomings of which the writer has not become aware. It is conceded that on the basis of the experience acquired during this work further attempts might be successful. Such hope, however, was insufficient assurance that the thesis research could be crowned by positive findings.

Out of this consideration—and it may be said that the decision was not an easy one—another substitution of the test organism was made. The choice of Calvatia maxima turned out to be a fortunate one. It could have been another failure, of course, and in that case undoubtedly a further attempt would have been made to find still another organism which would satisfy the requirements. If this research, therefore, may be found lacking a deliberately outlined experimental procedure which could be assessed beforehand to produce results, its unusual nature should be taken into consideration. On the other hand, it perhaps proves that persistence is as useful in research as experimental skill.

With the Calvatia maxima experiments it has been shown—and it is believed for the first time—that a tumor inhibitor originally observed in sporophore extracts could be produced at will and consistently in laboratory culture.

It can be presumed that the results of this study will serve the purpose of stimulating in vitro production of tumor inhibitors found in the sporophores of other Basidiomycetes.

Summary

Tests conducted at the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research disclosed that some aqueous extracts of Basidiomycete sporophores contained tumor-retarding substances. Experiments designed to develop methods of producing these biologically active substances under controlled conditions by laboratory cultures were described.

Boletus edulis var. pinicola, #288j, was the first organism investigated. Stationary and shake cultures were grown employing various media and environmental conditions. Preparations made from these cultures at times showed the presence of a tumor-retarding substance, but when duplication was attempted the results were inconsistent. Attempts to reach the goal with this organism were unsuccessful largely because of the difficulties of growing it with sufficient ease.

Collybia radicata var. furfuracea, #406f, was the next organism investigated. It was chosen chiefly because of its relatively fast growth rate. Various nutrilites, and carbon and nitrogen sources were studied as they affected the growth of mycelium and the elaboration of the tumor-retarding substances. Preparations made from cultures grown in media incorporating mannose or glucose at times indicated the presence of a tumor-retarding substance. However, these results could not be duplicated consistently. Attempts to reach the goal with this organism were also unsuccessful because the requirements of this organism with respect to the production of the tumor-retarding principle

could not be established with certainty in spite of the fact that the requirements for good mycelial growth were satisfied.

Calvatia maxima, #642, was the final organism studied. An investigation of various temperatures and growth periods disclosed that the tumor inhibitor which occurs in the sporophore was produced consistently by laboratory cultures.

BIBLIOGRAPHY

Albritton, E. C. (Editor) Standard values in nutrition and metabolism. Handbook of Biological Data (Second Part). Wright Air Development Center. Air Research and Development Command. U. S. Air Force. Wright-Patterson Air Force Base, Ohio. (1953).

Anchel, M. Identification of Drosophilin A as p-methoxy-tetrachlorophenol. Jour. Am. Chem. Soc. 74:2943 (1952).

Anchel, M., Hervey, A., Kavanagh, F., Polatnick, J., and Robbins, W. J. Antibiotic substances from Basidiomycetes III. Coprinus similis and Lentinus degener. Proc. Nat. Acad. Sci. 34:498-502, (1948).

Anchel, M., Hervey, A., and Robbins, W. J. Antibiotic substances from Basidiomycetes VII Clitocybe illudens. Proc. Nat. Acad. Sci. 36:300 (1950).

Anchel, M., Hervey, A., and Robbins, W. J. Antibiotic substances from Basidiomycetes. X. Fomes juniperinus Schrenk. Proc. Nat. Acad. Sci. 38:655-659 (1952).

Anchel, M., Hervey, A., and Robbins, W. J. Biological activity of p-methoxy-tetrachlorophenol. Mycologia 47:30-33 (1955).

Anchel, M., Polatnick, J., and Kavanagh, F. Isolation of a pair of closely related antibiotic substances produced by 3 species of Basidiomycetes. Archiv. Biochem. 25:208-220 (1950) .

Atkinson, N. Antibacterial activity in members of the higher fungi. 1. Cortinarius rotundisporus and Psalliota xanthoderma. Austral. Jour. Exp. Biol. Med. Sci. 24:169-173 (1946a).

Atkinson, N. Toadstools and mushrooms as a source of antibacterial substances active against Mycobacterium phlei and Bact. typhosum. Nature 157:441 (1946b).

Atkinson, N. Psalliotin, the antibiotic of Psalliota xanthoderma. Nature 174:598 (1954).

Bose, S. R. Antibacterial actions of 'polyporin' against typhoid, cholera, dysentery, and B. coli. Nature 156:171 (1945).

Bose, S. R. Antibiotic in a Polyporus. Nature 158:292-296 (1947).

Bottomley, W. B. Some accessory factors in plant growth and nutrition. Royal Society of London 88:237-247 (1914).

Bourquelot, E., et Hérissey, H. Chimie végétale. Sur la trehalase sa présence générale dans les Champignons. Comptes redus de L'academie des sciences 139:874-876 (1904)

Bourquelot, E., and Hérissey, H. Sur la tréhalâse, sa présence générale dans les Champignons. Bulletin de la Société mycologique de France 21:50-57 (1905).

Brunel, J. Antibiosis from Pasteur to Fleming. Contributions de l'Institut Botanique de l'Université de Montreal No. 67 (1951).

Burkholder, P. R., and Sinnott, E. W. Morphogenesis of fungus colonies in submerged shaken cultures. Am. J. Bot. 32:424-431 (1945).

Clarke, D. A. Mouse Sarcoma 180. Cancer Research, Supplement No. 3, 14 (1955).

Czapek, F. Untersuchungen über die Stickstoffgewinnung und Eiweissbildung der Pflanzen. I. Beiträge zur Chem. Physiol. und Path. 1: 538-560 (1902).

_____. Untersuchungen über die Stickstoffgewinnung und Eiweissbildung der Schimmelpilze. II. Über die Verwendbarkeit von Aminen, Amiden und Ammoniaksalzen zum Eiweissaufbau bei Aspergillus niger van Tiegh. Beiträge zur Chem. Physiol. ung Path. 2: 557-590 (1902a).

_____. Untersuchungen über die Stickstoffgewinnung und Eiweissbildung der Schimmelpilze. III. Die Verarbeitung von Nitro-und Hydrazinderivaten und von aromatischen Stickstoffverbindungen. Schlussbetrachtungen. Beiträge zur Chem. Physiol. und Path. 3: 47-66 (1903).

Davis, J. G., and Rogers, H. J. The effect of sterilisation upon sugars. Zentralblatt für Bakt., Parasit. und Infektionskrankheiten, Zweite Abt. 101: 102-110 (1939).

Derrick, M. J. Fundamental studies of the nutrition and physiology of wood destroying Basidiomycetes. Ph. D. Thesis, Syracuse University, (1949).

Doery, H. M., Gardner, J. F., Burton, H. S., and Abraham, P. Antibiotics from a Basidiomycete, Coprinus quadrifidus. Antibiotics and Chemotherapy 1:409-417 (1951).

Dorrell, W. W., and Page, R. M. The use of fragmented mycelial inoculum in the culture of fungi. Jour. Bact. 53:360-361 (1947).

Duggar, B. M. The principles of mushroom growing and mushroom spawn making. U. S. Dept. Agr., Bur. Pl. Ind., Bull. 85, 1-60 (1905).

Edgecombe, A. E. The effect of galactose on the growth of certain fungi. Mycologia 30:601-624 (1938).

Ehrlich, J., Anderson, L. E., Coffey, G. L., Hillegas, A. B., Knudsen, M. P., Koepsell, H. J., Kohberger, D. L., and Oyaas, J. E. Azaserine, a new tumor-inhibiting substance. Antibiotic studies of azaserine. Nature 173:72 (1954).

Englis, D. T., and Hanahan, D. J. Changes in autoclaved glucose. Jour. Am. Chem. Soc. 67:51-54 (1945).

Evans, R. J., and Butts, H. A. Inactivation of amino acids by autoclaving. Science 109:569-571 (1949).

Findlay, W. P. K. Effect of addition of sugar on rate of decay of wood. Ann. Applied Biol. 28:19-22 (1941).

Fries, N. Über die Bedeutung von Wuchsstoffen für das Wachstum verschiedener Pilze. Symb. Bot. Upsal. 3. (1938).

_____. Über die Sporenkeimung bei einigen Gasteromyceten und mykorrhizabildenden Hymenomyceten. Archiv für Mikrobiol. 12:266-284 (1941).

_____. Untersuchungen über Sporenkeimung und Mycelentwicklung bodenbewohnender Hymenomyceten. Symb. Bot. Upsal. 6:1-81 (1943).

_____. The nutrition of fungi from the aspect of growth factor requirements. Trans. Brit. Mycol. Soc. 30:118-134 (1948).

Guirard, B. M., Snell, E. E., and Williams, R. J. The nutritional role of acetate for lactic acid bacteria. I. The response to substances related to acetate. Archives of Biochem. 9:361-379 (1946a)

_____, _____, _____. The nutritional role of acetate for lactic acid bacteria. II. Fractionation of extracts of natural materials. Archives of Biochem. 9:381-386 (1946b).

Gussow, H. T., and Odell, W. S. Mushrooms and toadstools. F. A. Acland (Publisher) Ottawa, Canada (1927).

Hacskaylo, J., Lilly, V. G., and Barnett, H. L. Growth of fungi on three sources of nitrogen. Mycologia 46:691-701 (1954).

Hartelius, V., and Nielsen, N. Formation of yeast growth substances by heating sugar with ammonium hydroxide. Compt. Rend. Trav. Lab. Carlsberg. Ser. Physiol. 23:155-162 (1941).

Hawker, L. E. The effect of certain accessory growth substances on the sporulation of Melanospora destruens and of some other fungi. Ann. Bot. 50:699-717 (1936).

_____. Fungi and vitamins. Jour. Royal Coll. Sci. 14:65 (1944).

_____, and Chaudhuri, S. D. Growth and fruiting of certain ascomycetous fungi as influenced by the nature and concentration of carbohydrate in the medium. Ann. Bot. N. S. 10:185-194 (1946).

Hervey, A. H. A survey of 500 Basidiomycetes for antibacterial activity. Bull. Torrey Botan. Club 74:476-503 (1947).

Hodgman, C. D. Handbook of Chemistry and Physics, 32nd Edition. Chemical Rubber Publishing Co., Cleveland, Ohio (1950).

Hollande, A. C. Lyse massive des bacilles de Koch chez la Cobaye aprés traitement á la clitocybine. Pouvoir inhibiteur de ce produit vis-à-vis du bacille typhique, du colibacille, de Brucella abortus, etc. Compt. Rend. Acad. Paris 221:361-363 (1945).

Horr, W. H. Utilization of galactose by Aspergillus niger and Penicillium glaucum. Plant Physiol. 11:81-99 (1936).

How, J. E. The mycorrhizal relations of Larch. I. A study of Boletus elegans Schum. in pure culture. Ann. Bot. N. S. 4:135-150 (1940).

Humfeld, H. The production of mushroom mycelium (Agaricus campestris) in submerged culture. Science 107:373 (1948).

_____, and Sugihara, T. F. Mushroom mycelium production by submerged propagation. Food Technol. 3:355-356 (1949).

_____, _____. The nutrient requirements of Agaricus campestris grown in submerged culture. Mycologia 44:605-620 (1952).

Iwanoff, N. N. Über die Trehalose und Trehalase bei Myxomyceten. Biochem. Z. 162:455 (1925).

Jennison, M. W., Newcomb, M., and Henderson, R. Physiology of the wood-rotting Basidiomycetes. I. Growth and nutrition in submerged culture in synthetic media. Mycologia 47:275-304 (1955).

Kavanagh, F., Hervey, A., and Robbins, W. J. Antibiotic substances from Basidiomycetes. IV. Marasmius conigenus. Proc. Nat. Acad. Sci. 35:343-349 (1949).

_____, _____, _____, Antibiotic substances from Basidiomycetes. V. Poria corticola, Poria temuis, and an unidentified Basiciomycete. Proc. Nat. Acad. Sci. 36:1-7 (1950a).

Kavanagh, F., Hervey, A., and Robbins, W. J. Antibiotic substances from Basidiomycetes. VI. Agrocybe dura. Proc. Nat. Acad. Sci. 36:102-106 (1950b).

Khudiakov, J. P., and Vozniakovskaia, U. M. Pure cultures of mycorrhizal mushrooms. (Russian). Mikrobiologiia 20:13-19 (1951).

Klotz, L. J. Studies in the physiology of the fungi. XVI. Some aspects of nitrogen metabolism in fungi. Ann. Missouri Bot. Gard. 10:299-368 (1923).

Kluyver, A. J., and Perquin, L. H. C. Zur Methodik der Schimmelstoffwechseluntersuchung. Biochem. Z. 266:68-81 (1933).

LaFuze, H. H. Nutritional characteristics of certain wood-destroying fungi, Polyporus betulinus, Fr., Fomes pinicola (Fr.) Cooke, and Polystictus versicolor, Fr. Plant Physiol. 12:625-646 (1937).

Leonian, L. H., and Lilly, V. G. Studies on the nutrition of fungi. I. Thiamin, its constituents, and the source of nitrogen. Phytopath. 28:531-548 (1938).

_____, _____. Studies on the nutrition of fungi. IV. Factors influencing the growth of some thiamine-requiring fungi. Am. Jour. Bot. 27:18-26 (1940).

Lewis, I. M. The inhibition of Phytomonas malvaceara in culture media containing sugars. Jour. Bact. 19:423-433 (1930).

Lewis, R. W. An outline of the balance hypothesis of parasitism. The Am. Naturalist 87:273-281 (1953).

Lilly, V. G., and Barnett, H. L. Physiology of the fungi. McGraw Hill Book Co., Inc., New York, N. Y. (1951).

_____, _____. The utilization of sugars by fungi. W. Va. Univ. Ag. Exp. Sta. Bull. 362T (1953).

Lindeberg. G. Über die Wirkung von Biotin auf Marasmius androsaceus (L.) Fr. Arch. Mikrobiol. 12:58-62 (1941).

_____. Über die Physiologie ligninabbauender Boden-Hymenomyzeten. Studien an schwedischen Marasmius-Arten. Symb. Bot. Upsal. 8: 1-183 (1944).

_____. The effect of biotin and thiamin on the growth of Collybia dryophila Fr. Svensk. Bot. Tidskr. 40:63 (1946a).

_____. Thiamin and growth of litter decomposing hymenomycetes. Botaniska Notiser 1946:89-93 (1946b).

Löfgren, N., Lüning, B., and Hedström, H. The isolation of nebularine and the determination of its structure. Acta Chemica Scandinavica 8: 670-680 (1954).

Lutz, M. L. Sur la culture des Champignons Hyménomycètes en milieu artificiel. Compt. Rend. Acad. Sci. Paris 180:532-534 (1925).

Marczynski, R. Studies on the nutrition of Collybia velutipes (Curt.) Quel. (Homobasidiomycetes, Agaricales). Am. Midl. Natur. 30: 164-170 (1943).

Margolin, A. S. The effect of various carbohydrates upon the growth of some fungi. Ph. D. Thesis, West Virginia University (1942).

Mathieson, J. Antibiotics from Victorian Basidiomycetes. Austral. Jour. Exp. Biol. Med. Sci. 24:57-62 (1946).

Melin, E. Über die Mykorrhizenpilze von Pinus Silvestris L. und Picea Abies. (L.) Karst. (Vorläufige Mitteilung). Svensk. Bot. Tidskr. 15:192-203 (1921).

_____. Experimentelle Untersuchungen über die Birken- und Espenmykorrhizen und ihre Pilzsymbioten. Svensk. Bot. Tidskr. 17: 479-520 (1923).

_____. Der Einfluss von Waldstreuextrakten auf das Wachstum von Bodenpilzen, mit besonderer Berücksichtigung der Wurzelpilze von Bäumen. Symb. Bot. Upsal. 8 (1946).

_____, and Lindeberg G. Über den Einfluss von Aneurin und Biotin auf das Wachstum einiger Mykorrhizenpilze. Botaniska Notiser 1939:241-245 (1939).

_____, and Nilsson, H. Transport of labelled nitrogen from an ammonium source to pine seedlings through mycorrhizal mycelium. Svensk. Bot. Tidskr. 46:281-285 (1952).

_____, and Norkrans, B. Über den Einfluss der Pyrimidin- und der Thiazolkomponente des Aneurine auf das Wachstum von Wurzelpilzen. Svensk. Bot. Tidskr. 36:271-286 (1942).

_____, and Nyman, B. Weitere Untersuchungen über die Wirkung von Aneurin und Biotin auf das Wachstum von Wurzelpilzen. Arch. Mikrobiol. 11:318-328 (1940).

_____, _____. Über das Wuchsstoffbedurfnis von Boletus granulatus (L.) Fr. Arch. Mikrobiol. 12:254-259 (1941).

_____, Wiken, T., and Oblom, K. Antibiotic agent in the substrates from cultures of the genus Marasmius. Nature 159:840-841 (1947).

Myrbäck, K., and Örtenblad, B. Trehalose und Hefe. I. Biochem. Z. 288:329-337 (1936).

_____, _____. Trehalose und Hefe. II. Mitteilung: Trehalase-wirkung von Hefepräparaten. Biochem. Z. 291:61-69 (1937a).

_____, _____. Trehalose und Hefe. III. Biochem. Z. 292:230-233 (1937b).

Nielsen, N. and Eistrup, K. Formation of yeast growth substances by heating sugar, organic acids and ammonia. Compt. Rend. Trav. Lab. Carlsberg. Ser. Physiol. 23:79-92 (1939).

Norkrans, B. Studies in growth and cellulolytic enzymes of Tricholoma. Symb. Bot. Upsal. 11:1-127 (1950).

_____. The effect of glutamic acid, aspartic acid, and related compounds on the growth of certain Trichloloma species. Physiologia Plantarum 6:584-592 (1953).

Oddoux, L. Essai de culture de 508 especes d'homobasidiomycetes. Internat. Conf. on Sci. Aspects of Mushroom growing. Proc. 2, Mush. Sci. 2:28-39 (1953).

Pennington, L. H. Mycorrhiza-producing Basidiomycetes. Report Mich. Acad. Sci. 10:47-50 (1908).

Peterman, M. L., Hamilton, M. G., and Reilly, H. C. The basic proteins of Aspergillus fumigatus with tumor-inhibiting properties. Arch. Biochem. and Biophys. 37:117-130 (1952).

Porter, C. L. Mixed cultures of bacteria and fungi. Indiana Acad. Sci. Proc. 41:149-152 (1932).

Reilly, H. C., and Stock, C. C. Studies on a tumor-retarding agent produced by Aspergillus fumigatus. Cancer Research 11:366-369 (1951).

Rennerfelt, E. Die Entwicklung von Fomes annosus Fr. bei Zusatz von Aneurin und verschiedenen Extrakten. Svensk Bot. Tidskr. 38:153 (1944).

_____. Das Wachstum einiger Pilze aus Holzschliff bei verschiedener Temperatur. Archiv für Mikrobiol. 12:19-40 (1950).

Robbins, W. J. The assimilation by plants of various forms of nitrogen. Am. Jour. Bot. 24:243-250 (1937).

_____. Growth substances in agar. Am. Jour. Bot. 26:772-778 (1939).

Robbins, W. J. Further observations on factor Z. Bot. Gaz. 102:520-535 (1941).

_____. A survey of the growth requirements of some Basidiomycetes. Mycologia 42:470-476 (1950).

_____. Antibiotic substances produced by Basidiomycetes. Trans. N. Y. Acad. Sci. 16:39-43 (1953).

_____. Hervey, A., and Kavanagh, F. Production of antibiotic substances by Basidiomycetes. Jour. Bact. 51:410 (1946).

_____. Kavanagh, F., and Hervey, A. Antibiotics from Basidiomycetes. I. Pleurotus grieseus. Proc. Nat. Acad. Sci. 33:171-176 (1947a).

_____, _____, _____. Antibiotics from Basidiomycetes. II. Polyporus biformis. Proc. Nat. Acad. Sci. 33:176-182 (1947b).

_____, and Schmidt, M. B. Growth of excised roots of the tomato. Bot. Gaz. 99:671-728 (1938).

Roland, Jr., J. F. and Weiner, B. A. Bio-oxygenation of progesterone by mushrooms. Science 121:803-804 (1955).

Savage, G. M., and Vander Brook, M. J. The fragmentation of the mycelium of Penicillium chrysogenum and Penicillium notatum by a high speed blender and the evaluation of the blended seed. Jour Bact. 52: 385-391 (1946).

Schade, A. L. The nutrition of Leptomitus. Am. Jour. Bot. 27:376-384 (1940).

_____, and Thimann, K. V. The metabolism of the water mold Leptomitus lacteus. Am. Jour. Bot. 27:659-670 (1940).

Skoog, F. Substances involved in normal growth and differentiation of plants. Abnormal and pathological plant growth. Brookhaven National Laboratory, Upton, New York. BNL 258 (C-19). 303 pp. (1954).

Smith, A. H. Mushrooms in their natural habitats. Vol. 1, Text. 626 pp. Vol. 2, 231 stereochromes for Viewmaster. Sawyer's Inc., Portland, Oregon (1949).

Steinberg, R. Growth of fungi in synthetic nutrient solutions. Bot. Rev. 5: 327-350 (1939).

Stock, C. C. Aspects of approaches in experimental cancer chemotherapy. Am. Jour. Med. 8:658-674 (1950).

Stock, C. C. Experimental cancer chemotherapy. Advances in Cancer Research 2:425-492 (1954).

_____, Reilly, H. C., Buckley, S. M., Clarke, D. A., and Rhoads, C. P. Azaserine, a new tumor-inhibitory substance. Studies with Crocker mouse sarcoma 180. Nature 173:71 (1954).

_____, and Rhoads, C. P. Evaluation of chemotherapeutic agents. Columbia University Press, New York, N. Y. (1949).

_____, Sugiura, K., and Rhoads, C. P. The influence of antibiotic preparations on the viability and growth of sarcoma, melanoma and carcinoma in mice. Acta cancerologia 6:550 (1949).

Styer, J. F. Preliminary study of the nutrition of the cultivated mushroom. Am. Jour. Bot. 15:246-250 (1928).

_____. Nutrition of the cultivated mushroom. Am. Jour. Bot. 17: 983-994 (1930).

Tanner, F. W. Practical bacteriology, 2nd Edition. John Wiley and Sons, Inc., New York, N. Y. 235 pp. (1933).

Treschow, C. Nutrition of the cultivated mushroom. Dansk. Bot. Arkiv. 11:1-180 (1944).

Utech, N. M., and Johnson, J. The inactivation of plant viruses by substances obtained from bacteria and fungi. Phytopath. 40:247-265 (1950).

Van Niel, C. B. Recent advances in our knowledge of the physiology of microorganisms. Bact. Rev. 8:225-234 (1944).

Werkman, C. H. and Wilson, P. W. Bacterial physiology. Academic Press, Inc., New York, N. Y. 707 pp. (1951).

Wilkins, W. H. Investigations into the production of bacteriostatic substances by fungi; cultural work on Basidiomycetes. Trans. Brit. Mycol. Soc. 28:110-114 (1945).

_____. Investigations into the production of bacteriostatic substances by fungi; preliminary examination of more of the larger Basidiomycetes and some of the larger Ascomycetes. Ann. Applied Biol. 33:188-190 (1946a).

_____. Investigations into the production of bacteriostatic substances by fungi; preliminary examination of the fifth 100 species, all Basidiomycetes, mostly of the wood-destroying type. Brit. Jour. Exp. Path. 27:140-142 (1946b).

Wilkins, W. H. Investigations into the production of bacteriostatic substances by fungi; preliminary examination of the sixth 100 species, more Basidiomycetes of the wood-destroying type. Brit. Jour. Exp. Path. 28:53-56 (1947a).

_____. Investigations into the production of bacteriostatic substances by fungi; preliminary examination of the seventh 100 species, all Basidiomycetes. Brit. Jour. Exp. Path. 28:247-252 (1947b).

_____. Investigations into the production of antibacterial substances; preliminary examination of the eighth 100 species, all Basidiomycetes. Brit. Jour. Exp. Path. 28:416-417 (1947c).

_____. Investigations into the production of bacteriostatic substances by fungi; preliminary examination of the ninth 100 species, all Basidiomycetes. Brit. Jour. Exp. Path. 29:364-366 (1948).

_____, and Harris, G. C. M. Investigations into the production of bacteriostatic substances by fungi. VI. Examination of the larger Basidiomycetes. Ann. Applied Biol. 31:261-270 (1944).

Williams, R. J. Nutrilites. Science 67:607 (1928).

_____. Growth-Promoting nutrilites for yeasts. Biol. Rev., Cambridge Philosophical Soc. 16:49-80 (1941).

Zeller, S. M., and Schmitz, H. Studies in the physiology of the fungi. VIII. Mixed cultures. Ann. Missouri Bot. Gard. 6:183-192 (1919).

APPENDIX

Medium A

Agar	15.0 grams
$MgSO_4.7H_2O$	0.5 gram
KCl	0.5 gram
KH_2PO_4	1.0 gram
$FeSO_4 \cdot 7H_2O$	0.01 gram
Glucose	15.0 grams
Sucrose	15.0 grams
Bacto-Peptone	5.0 grams
Bacto-Yeast Extract	5.0 grams
Distilled water:	bring up to 1000 ml.
pH	5.6

Basal medium (Medium B)

Agar	15.0 grams
$MgSO_4.7H_2O$	0.5 gram
KCl	0.5 gram
KH_2PO_4	1.0 gram
$FeSO_4.7H_2O$	0.01 gram
Glucose	15.0 grams
Sucrose	15.0 grams
Bacto-Peptone	5.0 grams
Distilled Water:	bring up to 1000 ml.
pH	5.6

Sodium caseinate medium (Medium G)

Sodium caseinate	2.0 gram
Glucose	1.0 gram
K_2HPO_4	0.2 gram
$MgSO_4.7H_2O$	0.2 gram
$FeSO_4$ (0.1% solution)	1.0 ml.
Peptone	1.0 gram
Glycerine	5.0 gram
Agar	15.0 gram
Distilled water to	1000 ml.

pH not adjusted, usually 7.3 before autoclaving.

Khudiakov's medium (Medium J)

Glucose	15.0 grams
NH_4NO_3	1.0 gram
KH_2PO_4	1.0 gram
$MgSO_4$	0.5 gram
$CaCl_2$	0.1 gram
NaCl	0.1 gram
Casein hydrolysate containing a 0.5% tryptophan solution	5.0 grams
Agar	15.0 grams
$FeCl_2$	0.6 mg.
$CuSO_4$	0.23 mg.
$Na_2B_4O_7$	0.045 mg.
Na_2MoO_4	0.043 mg.
$MnCl_2$	0.046 mg.
$ZnSO_4$	4.300 mg.
Nicotinic acid	1.000 mg.
Para-aminobenzoic acid	1.000 mg.
Riboflavine	1.000 mg.
Calcium pantothenate	1.000 mg.
Biotin	1.000 mg.
Thiamine	1.000 mg.
Distilled water:	bring up to 1000 ml.

Note: The agar content was modified to read 20.0 grams, to give a firmer more solid medium when this was desired.

The casein hydrolysate was modified to read 5.00 ml. casein hydrolysate and 0.025 gram tryptophan.

Carrot Medium

Carrot slices	200 grams
Distilled water	500 ml.

Steam for 30 minutes, then filter through cheesecloth.

Add distilled water to bring volume up to 1000 ml.

Agar	20 grams

Fries Mineral Medium

Ammonium tartrate	5.0 grams
Ammonium nitrate	1.0 gram
KH_2PO_4	1.0 gram
NaCl	0.1 gram
$CaCl_2$ (anhydrous)	0.1 gram
$MgSO_4.7H_2O$	0.5 gram
Sucrose	15.0 grams
Distilled water:	bring up to 990 ml.

Autoclaved at 15 lbs. pressure for 12 minutes.

10.0 ml. of the following vitamin solution added after cooling:

Inositol	40 mg.
Para-aminobenzoic acid	0.3 mg.
Thiamine HCl	0.3 mg.
Pyridoxine HCl	0.4 mg.
Biotin	0.005 gamma
Distilled water:	bring up to 1000 ml.

Lindeberg's Nutrient Solution B

Glucose	20.0 grams
Ammonium tartrate	5.0 grams
KH_2PO_4	1.0 gram
$MgSO_4.7H_2O$	0.5 gram
$FeCl_3$ solution (Fe conc. 1/500)	0.5 ml.
$ZnSO_4$ solution (Zn conc. 1/500)	0.5 ml.
$MnCl_2$ (0.1 M solution)	0.5 ml.
$CaCl_2$ (0.1 M solution)	5.0 ml.
Distilled water	995.0 ml.

pH after autoclaving is 5.5

Typical analysis of Bacto-Yeast Extract*

Per Cent	
Ash	10.1
Total N	9.18
Chloride	0.190
Total Suphur	1.39
PPM	
Lead	16.00
Arsenic	0.11
Manganese	7.8
Zinc	88.00
Copper	19.00
Per Cent	
Phosphorus	0.89
Iron	0.028
SiO_2	0.052
Potassium	0.042
Sodium	0.32
Magnesium	0.030
Calcium	0.0406
Arginine	0.78
Aspartic acid	5.1
Glutamic acid	6.5
Glycine	2.4
Histidine	0.94
Isoleucine	2.9
Leucine	3.6
Lysine	4.0
Methionine	0.79
Phylalanine	2.2
Threonine	3.4
Tryptophane	0.88
Tyrosine	0.60
Valine	3.4
Micrograms per gram	
Pyridoxine	20.0
Biotin	1.4
Thiamine	3.2
Nicotinic acid	279.00
Riboflavine	19.00
Folic acid	0.3

*Analysis furnished by Difco Laboratories, Incorporated, Detroit 1, Michigan

Typical analysis of Bacto-Peptone*

Total Nitrogen	16.16%
Primary Proteose N	0.06%
Secondary Proteose N	0.68%
Peptone N	15.38%
Ammonia N	0.04%
Free Amino N (Van Slyke)	3.20%
Amide N	0.49%
Mono-amino N	9.42%
Di-amino N	4.07%
Tryptophane	0.29%
Tyrosine	0.98%
Cystine (Sullivan)	0.22%
Organic Sulphur	0.33%
Inorganic Sulphur	0.29%
Phosphorus	0.22%
Chlorine	0.27%
Sodium	1.08%
Potassium	0.22%
Calcium	0.058%
Magnesium	0.056%
Manganese	nil
Iron	0.0033%
Ash	3.53%
Lead	15.00 ppm
Arsenic	0.09 ppm
Zinc	18.00 ppm
Copper	17.00 ppm
SiO_2	0.042%
Arginine	8.00%
Aspartic acid	5.90%
Glutamic acid	11.00%
Glycine	23.00%
Histidine	0.96%
Isoleucine	2.00%
Leucine	3.50%
Lysine	4.30%
Methionine	0.83%
Phenylalanine	2.30%
Threonine	1.60%
Valine	3.20%
Pyridoxine	2.50 gamma/gm.
Biotin	0.32 gamma/gm.
Thiamine	0.50 gamma/gm.
Nicotinic acid	35.00 gamma/gm.
Riboflavine	4.00 gamma/gm.

*Analysis furnished by Difco Laboratories, Incorporated, Detroit 1, Michigan.

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Thesis

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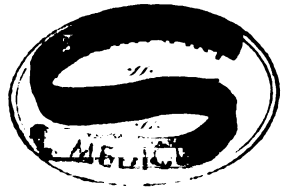

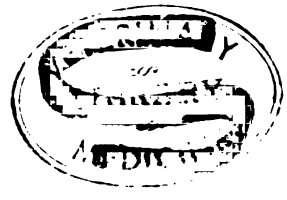

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